Anti-biofilm activity of plants used in Ayurvedic medicine and their molecular mechanisms of action on *E. coli* biofilms

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Amita Bhatti

President of the Humboldt-Universität zu Berlin

Prof. Dr.-Ing. Dr. Sabine Kunst

Dean of the Faculty of Life Sciences

Prof. Dr. Bernhard Grimm

Reviewers: 1) Prof. (Dr.) Regine Hengge

2) Prof. (Dr.) Kürsad Turgay

3) Prof. (Dr.) Natalia Tschowri

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“Dedicated to

my loving parents

and family”

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Zusammenfassung

Biofilme sind Ansammlungen von Mikroben, die in extrazelluläre polymere Substanzen (EPS) eingebettet sind, die aneinander und/oder an Oberflächen haften. Bakterien neigen dazu, in der Natur als Biofilme zu wachsen, die emergente Eigenschaften besitzen, die sie von ihren planktonischen Gegenstücken unterscheiden. Je nach Art der Bakterien haften sie mit adhäsiven Organellen wie Fimbrien/Pili sowie Flagellen an Oberflächen. Biofilme entwickeln sich zu dynamischen dreidimensionalen Strukturen.

Die Biofilmbildung, insbesondere die Matrixproduktion, wird durch komplexe molekulare regulatorische Netzwerke reguliert. Amyloide Fasern, die Hauptbestandteile der extrazellulären Matrix vieler bakterieller Biofilme sind, sind diverse und abundante extrazelluläre Proteine, die aus Untereinheiten zusammengesetzt sind, sich als β-Faltblätter formen. In *E. coli* bestehen diese amyloiden Curli-Fasern aus den Genprodukten der Gene *csgBA*, wobei CsgA die Hauptkomponente und CsgB eine Nebenkomponente ist, die als Nukleator für die Polymerisation der CsgA-Fasern dient. Das *csgDEFG*-Operon kodiert CsgD, den positiven Transkriptionsregulator des Systems, sowie das Sekretionssystem der Curli-Fasern. Darüber hinaus produziert *E. coli* pEtN-Cellulose, wobei die Operons *bcsQABZC* und *bcsEFG* an der Synthese und Translokation von pEtN-Cellulose beteiligt sind. Sowohl die Produktion der Curli- als auch die der pEtN-Cellulosefasern steht unter der Kontrolle von CsgD, das selbst wiederum durch ein komplexes regulatorisches Netzwerk reguliert wird, an dem der Sigma-Faktor der stationären Phase RpoS (σ^S), die sekundären Botenstoffe c-di-GMP sowie (p) ppGpp und mehrere Regulatoren der Stressantwort auf Zellhüllstress beteiligt sind.

Antibiotikaresistenz/-toleranz und Evasion des menschlichen Immunsystems sind zentrale Probleme im Zusammenhang mit persistierenden chronischen Infektionen, die im Zusammenhang mit Biofilmen stehen. Daher ist die Notwendigkeit einer alternativen Behandlung dringend erforderlich. Von hunderten Pflanzenextrakten und reinen Inhaltsstoffen von Pflanzen, ist bereits bekannt, dass sie entweder die Biofilmbildung verschiedener Bakterienarten hemmen und/oder in einigen Fällen sogar bereits existierende Biofilme beseitigen können. Die diesen Aktivitäten zugrunde liegenden molekularen Mechanismen wurden jedoch bisher kaum untersucht. In den letzten Jahren hat die AG Hengge begonnen, die Anti-Biofilm-Mechanismen von Pflanzenextrakten zu untersuchen, die traditionelle Heilpflanzen aufweisen, die gegen chronische bakterielle Infektionen eingesetzt werden, und dabei festgestellt, dass die Bildung Amyloider Fasern, sowie regulatorische Prozesse als Ziele für Anti-Biofilm-Stoffe dienen können.

In dieser Arbeit wurden 10 von 19 Pflanzen ausgewählt, welche anfangs aufgrund ihrer Verwendung gegen neurodegenerative Erkrankungen oder zur Gedächtnisförderung in der ayurvedischen Medizin ausgewählt wurden. Diese Auswahl basierte auf dem Präzedenzfall des Grüntee-Polyphenols EGCG, von dem festgestellt wurde, dass es die Bildung von Amyloid-Alzheimer-A-beta-Plaques stört (Ehrnhoefer *et al.* 2008) und sich dann auch als wirksames Mittel gegen die Bildung von Amyloid-Fasern von *E. coli* Biofilmen erwies (Serra et al. 2016). Diese 10 Pflanzen wurden ausgewählt, weil sie eine deutliche Hemmung der Produktion von Curli- und pEtN-Cellulosefasern oder nur von Curli-Fasern von *E. coli* Makrokolonie Biofilmen zeigten. Eine Kombination von mikrobiologischen, molekularbiologischen und enzymatischen Experimenten wurde verwendet, um die Aktivitäten der Pflanzenextrakte sowie einiger ihrer reinen Bestandteile weiter zu charakterisieren. Um ihre Wirkungsweise auf *E. coli* aufzuklären, wurde eine große Reihe molekularer Reporter eingesetzt. Direkte Auswirkungen auf die Bildung der Amyloid-Fasern wurden durch Experimente mit gereinigten Curli-Untereinheiten (CsgA) gezeigt. Um die Wirkung gegen Biofilme eines breiteres Spektrums von Bakterien zu testen, wurden auch relevante gramnegative Krankheitserreger (EAEC, UPEC, *Pseudomonas aeruginosa*) und grampositive Bakterien (*Bacillus subtilis*, *Staphylococcus aureus*) hinsichtlich der Makrokoloniebildung sowie submersen Biofilmbildung in Gegenwart von aktiven Pflanzenextrakten untersucht.

Das wichtigste Ergebniss dieser Studie ist, dass es kein „Allheilmittel“ gibt, das effektiv gegen die verschiedenen Biofilmzusammensetzungen und -strukturen wirken kann, die von verschiedenen Bakterienarten produziert werden. Pflanzen enthalten vielmehr Cocktails aus Wirkstoffen, um synergistisch auf verschiedene molekulare Ziele zu wirken und unter verschiedenen Bedingungen mit verschiedenen Bakterienarten umzugehen. Es wurde festgestellt, dass fast alle Pflanzenextrakte die CsgA-Amyloidogenese hemmen, aber auch auf zusätzliche Ziele einwirken. So beeinflussten *Bacopa monnieri*, *Glycyrrhiza glabra* und *Aegle marmelos* auch die Curli- und pEtN-Cellulose-Gene signifikant, indem sie *csgB* und *dgcC* über CsgD herunterregulierten. Darüber hinaus wurde festgestellt, dass *Bacopa monnieri* die Expression von flagellaren Genen in *E. coli* hochreguliert - dies ist eine neue Anti-Biofilm-Strategie, die zuvor nicht berücksichtigt wurde. Überraschenderweise wurde auch festgestellt, dass ein Pflanzenextrakt (*Glycyrrhiza glabra*), der die Biofilmbildung eines Bakterienstamms, wie dem Kommensal *E. coli* K-12 hemmt, während die Biofilmbildung bei uropathogenem *Escherichia coli* (UPEC) gefördert wird, was bedeutet, dass stammspezifische Effekte aufgrund der unterschiedlichen Zusammensetzung der Matrix innerhalb derselben Bakterienart auftreten können. Einige der Pflanzenextrakte, zum Beispiel *Glycyrrhiza glabra* und *Garcinia*

mangostana waren hochgiftig für grampositive Bakterien und andere reduzierten das Wachstum der Bakterien. Es konnte daher geschlussfolgert werden, dass grampositive Bakterien gegenüber den meisten Pflanzenextrakten empfindlicher sind, höchstwahrscheinlich aufgrund ihrer unterschiedlichen Zusammensetzung und Struktur der Zellwände. Um mit bakteriellen Biofilmen umzugehen, die aus mehreren Arten bestehen, kann eine Strategie mit kombinierten Pflanzenextrakten funktionieren, bei der, aber noch weitere Forschung und ein besseres Verständnis erforderlich sind, um diesen Ansatz weiterzuverfolgen.

Summary

Biofilms are an aggregation of microbes embedded in extracellular polymeric substances (EPS) that adhere to each other and/or surfaces. Bacteria tend to thrive in nature as biofilms, which has emergent properties that differentiate them from their planktonic counterparts. Bacteria attach to the surfaces with some adhesive organelles such as fimbrial adhesins/pili and flagella, depending on the type of bacterial species. Biofilms develop into mature 3D structures, which are dynamic in nature.

Biofilm formation, in particular matrix production, is regulated by complex molecular regulatory networks. Amyloid fibers, which are major extracellular matrix components of many bacterial biofilms, are diverse and abundant extracellular proteins assembled from subunits that are arranged in β-sheets. In *E. coli*, amyloid curli fibers are produced from genes *csgBA*, with CsgA being the major component and CsgB the minor component that 'nucleates' polymerization of CsgA fibers. The *csgDEFG* operon encodes CsgD, the positive transcriptional regulator of the system, as well as the curli secretion system. In addition to this, *E. coli* produces pEtN-cellulose, with the *bcsQABZC* and *bcsEFG* operons being involved in synthesis and translocation of pEtN-cellulose. Both curli and pEtN-cellulose production are under the control of CsgD, which in turn is regulated by a complex regulatory network that involves the stationary phase sigma factor RpoS (σ^S), the second messengers c-di-GMP and (p)ppGpp and several regulators of cell envelope stress responses.

Antibiotic resistance/tolerance and evasion from the human immune system are major causes of concern associated with biofilm-related persistent chronic infections. So, the need for an alternative source of treatment is urgently needed. Hundreds of plant extracts and pure compounds derived from plants have been reported to either inhibit biofilm formation of different bacterial species and/or in some cases even to eradicate already established biofilms. However, the molecular mechanisms underlying these activities have hardly been studied. During the past years, the Hengge group has started to unravel anti-biofilm mechanism of compounds that occur in traditional medicinal plants used against chronic bacterial infections and found that amyloid formation as well as regulatory processes can serve as targets for anti-biofilm compounds.

In this study, 10 plants were selected from 19 plants initially chosen based on their use against neurodegenerative diseases or for memory promotion in Ayurvedic medicine. This rationale was based on the precedent of the green tea polyphenol EGCG, which was first found

to interfere with amyloid Alzheimer beta plaque formation (Ehrnhoefer et al. 2008) and then also proved a potent agent against amyloid fibre formation in *E. coli* biofilms (Serra et al. 2016). These 10 plants were chosen because they showed clear inhibition of curli fiber and pEtN-cellulose production or against curli fibers only in *E. coli* macrocolony biofilms. A combination of microbiological, molecular biological, and enzymatic assays and experiments were then used to further characterize the activities of the plant extracts as well as some of their pure constituents. In order to elucidate their mode of action on *E. coli*, a large series of molecular reporters were used. Direct effects on amyloidogenesis were shown using an assay for purified curli subunits CsgA. To test anti-biofilm effects on a wider range of bacteria, also some relevant Gram-negative pathogens (EAEC, UPEC, *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*) were grown in macrocolony biofilms and submerged biofilms in the presence of active plant extracts.

The major findings of this study are that there is not one single "magic bullet" that can effectively work against the diverse biofilm compositions and structures produced by different bacterial species. Rather, plants contain cocktails of active compounds to act synergistically on different molecular targets and to deal with different bacterial species under different conditions. Nearly all plant extracts were found to inhibit CsgA amyloidogenesis, but also acted on additional targets. Thus, *Bacopa monnieri*, *Glycyrrhiza glabra* and *Aegle marmelos* also affected the curli and pEtN-cellulose genes significantly by downregulating *csgB* and *dgcC* via the CsgD regulator. In addition, *Bacopa monnieri* was found to upregulate flagellar gene expression in *E. coli* - this is a new anti-biofilm strategy that had not considered before. Surprising, it was also noticed that one plant extract (*Glycyrrhiza glabra*), which is inhibiting biofilm formation of one bacterial strain such as commensal *E. coli* K-12, is promoting biofilm in uropathogenic *Escherichia coli* (UPEC), which means effects can be strain-specific because of the diversity of composition of the matrix within the same bacterial species. Some of the plant extracts, for instance *Glycyrrhiza glabra* and *Garcinia mangostana,* were highly toxic to Gram-positive bacteria and others reduced bacterial growth. So, it could be concluded that Gram-positive bacteria are more sensitive to the majority of plant extracts, most likely because of their difference in cell wall composition and structure. So, to deal with bacterial biofilms where multiple species are involved, a strategy of combining different plant extracts may work, but definitely, more research and understanding are still required to follow up on this approach.

Abbreviations

A	Ampere
A-site	Active site
aa	Amino acid
Amp	Ampicillin
APS	Ammonium persulphate
bp	Base pair
c-di-GMP	Cyclic di-guanosine monophospahte
cAMP	Cyclic adenosine monophosphate
Cm	Chloramphenicol
CR	Congo red
Da	Dalton
ddH_2o	Double distilled water
DGC	Diguanylate cyclase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	1,4-dithioreitol
EAEC	Enteroaggregative *Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
g	Gram
hr	Hour
HRP	Horseradish peroxidase
IPTG	Isopropyl-beta-D-thiogalactopyranoside
L	Liter
LB	Lysogeny broth
LBon	Lysogeny broth without salt
m	Milli
M	Molar
min	Minute

mRNA	Messenger RNA
N-terminal	Amino terminal
OD	Optical density
ONPG	Ortho-nitrophenly-beta-D-pyranoside
ORF	Open reading frame
PDE	Phosphodiesterase
pEtN	Phosphoethanolamine
RNA	Ribonucleic acid
RNAP	RNA-polymerase
rpm	Rotations per minute
RT	Room temperature
SDS	Sodiumdodecylsulphate
TEMED	Tetramethylenthyldiamine
TRIS	Trishydroxymethylaminomethane
UPEC	Uropathogenic *Escherichia coli*
V	Volt
X-Gal	5-bromo-4-chloro-3-indol-beta-D-galactopyranoside
EPS	Extracellular polymeric substances
eDNA	Extracellular Deoxyribonucleic acid
3D	Three dimensional

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1. Introduction

1.1. Bacterial Biofilms: History vs current studies

To avoid incoherent terminology of biofilm by various scientists from different fields such as medicine, environment, agriculture, biotechnology, pharmacology, etc., IUPAC recommends the definition of biofilm as '***aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhere to each other and/or to a surface***' (Michel et al., 2012). Biofilm aggregates can either be formed on the surfaces, in which only one layer is directly attached to the surface which might be providing nutrients to the growing biofilm, or flocs which are free-floating biofilm aggregates that are not attached with any surface/substratum(Flemming et al., 2016). Socio-physio interactions among the cells in biofilm and the properties of the extracellular matrix make it distinct from their free-living counterparts. Consequently, the emergent properties of biofilms are contrasting from the planktonic bacteria (Flemming et al., 2016).

The observations of aggregates of biofilm are as old as Microbiology, Anthony van Leeuwenhoek (1632-1723), observed the biofilm the very first time using his primitive microscope. He saw aggregates of cells in the "scurf of teeth" and from "particle scraped off his tongue" (De Kruif, 1996) from his mouth sample. Louis Pasteur observed such aggregates in the process of fermentation and sketched them (Hoiby, 2017). In the 1930s, environmentalist, Henrici, found that water bacteria are not free-living rather they grow attached on submerged surfaces by using direct microscopic techniques (Zobell & Allen, 1935).

In the history of Medical Microbiology, Costerton introduced the terminology "**Biofilm**" the very first in 1985, and with his co-workers, he demonstrated that the cells in biofilms of *Pseudomonas aeruginosa* confer tobramycin resistance in contrast to free-floating cells (Nickel, Ruseska, Wright, & Costerton, 1985). Before that, he also used the term 'Biofilm' in one of his technical microbiology reports (McCoy, Bryers, Robbins, & Costerton, 1981). There are only 3 publications on PubMed on biofilms but later in the following decade, the number increased from 100s to 1000s. From 1985 to 1995 there were 762 publications. In the following decade, the number hiked to 4805. And most surprisingly, I found that only in the year 2018, there were 3916 publications on biofilms on PubMed (Recorded on 30th July 2019). So, it can be concluded that biofilm study in the current scenario is one of the most intriguing fields among microbiologists, environmentalists, biotechnologists, marine biologists, pharmacists, etc.

Though the aggregated bacteria observed and sketched in the late 16th century by Leeuwenhoek yet its existence has been documented in the ancient fossils records, particularly in hydrothermal records (Rasmussen, 2000; Reysenbach & Cady, 2001; Reysenbach, Ehringer, & Hershberger, 2000; Westall, 2005). The initial basic studies of biofilm in the field of environment later opened a way to understand and characterize biofilms on medical devices and in chronic infections associated with biofilms. Many of the physiological characteristics of biofilms in their natural environment are similar to biotic surfaces (animal and or human host) such as adherence to the surface and posing antibiotic tolerance to the cells (Hall-Stoodley, Costerton, & Stoodley, 2004).

What is the purpose of bacteria to live together in a biofilm? In general, it has been suggested that it a key factor that confers survival strategies to help them to survive in a wide range of environmental conditions. It protects the cells under unfavorable environmental conditions and it also helps in dispersions. Following are the various protection which biofilms are conferring against various hostile conditions which are hypothesized/confirmed by many researchers:

1. UV irradiation (de Carvalho, 2017; Espeland & Wetzel, 2001)
2. Metal toxicity (Grujic, Vasic, Comic, Ostojic, & Radojevic, 2017; Teitzel & Parsek, 2003)
3. Acid exposure (McNeill & Hamilton, 2003) (Akinbobola, Sherry, McKay, Ramage, & Williams, 2017)
4. Dehydration and salinity (Le Magrex-Debar, Lemoine, Gelle, Jacquelin, & Choisy, 2000) (van de Mortel & Halverson, 2004)
5. Phagocytosis (Hoiby, Bjarnsholt, Givskov, Molin, & Ciofu, 2010; Leid, Shirtliff, Costerton, & Stoodley, 2002; Y. Zhao, Song, Wang, Zhou, & Ren, 2017)
6. Several antibiotics and antimicrobial agents (Hoiby et al., 2010; Mah & O'Toole, 2001)

Because of such emergent properties of biofilms in contrast to their counterpart, free-floating individual cells, biofilms can pose serious problems in the natural environment and can also cause chronic infectious diseases in plants, animals, and humans. This review of literature will mainly focus on approaches for combating biofilms associated with bacterial infections exploring different anti-biofilm agents and their mode of actions. It will also discuss briefly the biofilm formation, the molecular mechanism of biofilm formation using *E. coli* as a study model, mechanism of antibiotic resistance and tolerance in biofilms, chronic infections associated with biofilms following detailed discussion about recent techniques to deal with it.

1.1.1. Bacterial biofilms in natural environments

Bacteria can live in almost every kind of conditions and surfaces such as hydrothermal vents (Li Y, 2018) (Meier DV, 2019), marine environment (Louca S, 2016), soil, air to colonizing on other living organisms such as plants and animals (Bogino, Oliva Mde, Sorroche, & Giordano, 2013; Tremblay, Hathroubi, & Jacques, 2014). Bacterial lifestyle has recently come into consideration. Historically, they were considered as individuals growing and multiplying without interacting with each other.

From the last couple of decades, it has been found that most bacterial species survive in the natural environment by forming structural communities known as biofilms. These structural and functional communities can develop on any kind of surface such as indwelling medical devices, living tissues, water piping (both potable or industrial), or even in natural aquatic ecosystems. The structure of biofilm varies from photosynthetic mats (Reysenbach & Cady, 2001) to mushroom-like structures (J. K. Miller et al., 2012) or many more. These variations depend on several factors such as changes in nutrient conditions and hydrodynamics etc. (Stoodley, Dodds, Boyle, & Lappin-Scott, 1998).

The developmental stages of biofilms, both in the natural and anthropogenic environment have been deeply investigated since biofilm research become a hot topic among microbiologists and various other researchers from different fields. These complex and differentiated structures are observed and studied both *in situ* and/or the artificial environment of laboratories (Fernández-Barat et al., 2018). The initial step in the development of biofilm is the attachment of bacterial cells onto the surfaces followed by maturation of complex structure and finally shedding the part of it for dispersal (Donlan, 2002). Initial attachment of the cells onto the surface is loose and reversible followed by tight and irreversible attachment. Several known bacterial organelles or secreted adhesins are facilitating the initial irreversible attachment of bacterial planktonic cells such as fimbrial adhesins/pili (Pratt & Kolter, 1998) as in the case of *E. coli,* and UPEC (Uropathogenic *E. coli*) and flagella and Type IV pilli in *Pseudomonas* aeruginosa (George A O'Toole & Kolter, 1998). In the case of *Staphylococcus aureus*, dozens of specific staphylococcal surface proteins, MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) are expressed that can bind to human plasma (Otto, 2018). Atl (bifunctional enzyme acting as amidase and glucosaminidase after catalytic cleavage) and wall teichoic acids in *S. aureus* also have implications in binding to surfaces (Bose, Lehman, Fey, & Bayles, 2012). Bap biofilm-associated-protein is another non-fimbrial adhesion that is known to be secreted via Type 1 secretion system that is involved in many Gram-positive (Cucarella

et al., 2001) and Gram-negative bacteria (Noori, Rasooli, Owlia, Mousavi Gargari, & Ebrahimizadeh, 2014).

After irreversible attachment of bacterial cells onto biotic and/or abiotic surfaces, cells mature to form a biofilm to keep them together, so, to keep the cells in close association with each other, antigen Ag43 in *E. coli* (surface autotransporter protein), promotes cell to cell adhesins, involved in auto-aggregation and flocculation of the cells in a liquid medium and helps in building interspecies communities (Kjaergaard, Schembri, Hasman, & Klemm, 2000). During their maturation phase, bacterial cells produce a large amount of extracellular matrix that makes them distinct from their counterpart, free-living cells, and acts as cementing material of biofilm complex architectures (Beloin, Roux, & Ghigo, 2008). The basic constituents of biofilm are water, exopolysaccharides polymers, proteins, nucleic acids, lipids, nutrients, and metabolites (Beloin et al., 2008). Although there are some universal similarities among all the bacterial biofilm architecture yet every complex and differentiated biofilm structures are unique among different bacterial species (Tolker-Nielsen & Molin, 2000). Biofilm structures are highly dynamic, constantly breaking and building where dissociation of daughter cells from the biofilm can be done by actively growing cells or just by shearing of a small portion of the biofilm aggregate (Donlan, 2002).

1.1.2. Heterogeneity and inter/intra communication within natural bacterial biofilms

Biofilms are heterogeneous as they form different zones based on differences in matrix production and physiology (R. Hengge, 2020). In *E. coli* microcolony biofilms, there is constant availability of nutrients at the lower phase next to the agar and thus helps the cells to keep growing and producing a network of non-motility-associated intertwined flagella which remains free of the extracellular matrix. In contrast, the upper layer is away from nutrients and thus the cells enter into the stationary phase and produce extracellular matrix rich in amyloid fibrils and phosphoetanolamine cellulose (pEtN-cellulose) (D. O. Serra, Richter, & Hengge, 2013; Diego O. Serra, Richter, Klauck, Mika, & Hengge, 2013; Thongsomboon et al., 2018).

The proximity of cells within the exopolysaccharide confers various benefits to the community and protects it from various physical and chemical dangers around them. In addition to this, biofilms provide a conducive environment for the horizontal gene transfer such as the exchange of plasmids (extrachromosomal DNA) and it has been found that the rate of transfer of plasmids within biofilms is higher than in free-living bacteria (Hausner & Wuertz, 1999). Transfer of antibiotic resistance genes by horizontal gene transfer within the biofilm is a matter of concern

in these complex architectures. Horizontal gene transfer within the biofilm can occur by classical phenomena such as conjugation (Ghigo, 2001), transformation (Lattar et al., 2018), and bacteriophage infections (Solheim, Sekse, Urdahl, Wasteson, & Nesse, 2013). Recently, DNA containing membrane vesicles is also known for supporting the genetic exchange between the bacterial cells in biofilms (Biller et al., 2014). All such activities within the biofilm are supporting the whole community to adapt in the new changing environment such as the presence of antimicrobial agents in their vicinity, heavy metals, or any other chemical pollutant which is otherwise toxic for planktonic, free-living cells. In other words, biofilm is not only providing bacterial tolerance against antibiotics but also providing niche for the exchange of genes that could confer antibiotic resistance. It has already been known that quorum sensing (cell-to-cell signaling) plays an important role in bacterial attachment and 3-D (3-dimensional) biofilm developmental structures (Davies et al., 1998). Biofilms can also act as reservoirs of quorum sensing molecules (both *in vivo* and *in vitro*) (Stickler, Morris, McLean, & Fuqua, 1998; Taghadosi, Shakibaie, & Masoumi, 2015). The actual role of quorum sensing molecules within the biofilm are still under investigation.

In a natural environment, bacteria are surviving and competing with other species for sharing the same space for the requirement of nutrients, oxygen, and space. Under laboratory conditions, dual-species biofilm has uncovered many aspects of interspecies associations within the multispecies biofilm. Schaefer et al in 2013, found that *Salmonella* species in a monoculture develops a very loose and easily removable biofilm but developed highly structured biofilms when grown on a developed mixed culture biofilm (with *Paenibacillus favisporus*, *P. cineris*, *Paenibacillus* sp., *Bacillus* sp. and *Enterococcus mundtii*) (Schaefer, Brözel, & Venter, 2013). Similar results have also been found in a study conducted by Jones and Bradshaw in a laboratory reactor and found that *Salmonella enteritidis* when passed from an already established biofilm of *Klebsiella pneumoniae*, it rapidly produces biofilm (K. Jones & Bradshaw, 1997). In addition to this, they also found that in a co-culture, they are actively producing metabolically active cells and the level of attachment also increases in comparison to a single culture. The growth rate and utilization of nutrients of *Klebsiella pneumoniae* are higher in a mixed biofilm and it also provides an anchor for other organisms to attach to them (K. Jones & Bradshaw, 1997). Most recently, Yamakawa et al in 2018, assessed the properties of co-cultured biofilm of *Listeria monocytogenes* and *Pseudomonas aeruginosa* at low temperatures and found that they become much more resistance to food-grade antibacterial compounds (Yamakawa, Tomita, & Sawai, 2018).

Biofilms are not only providing protections to bacterial communities from UV irradiation, metal toxicity, acid exposure, dehydration and salinity, phagocytosis, and several antibiotic and antimicrobial agents to the bacterial cells, there are lots of interactions and signaling which are happening within microbial biofilm communities that are increasing their chances to adapt in changing environments. Insight into the proximity and inter/intracellular interactions within the biofilm bringing more challenges for the researchers to deal with them in their natural and/or changing environment.

1.1.3. Biofilm associated infections: cause of concern

Biofilms are a cause of concern in many fields such as the food industry, dairy industry, wastewater treatment plants, water purification units, water supplying piping, marine world and human and animal health. Their community behavior and inter and intra communications within biofilm *in situ* are making the situations tougher to deal with them. This review will only deal with biofilm-associated infections, their cause, and new current strategies.

Studies already suggested that persistent chronic infections are a major concern as they are difficult to treat with antibiotics (Yamakawa et al., 2018). Numbers of mechanisms are thereby which bacteria can evade from the immune system and antibiotics (Munita & Arias, 2016). One such major mechanism is by producing extracellular matrix and forming 3D structural communities, biofilms.

Pseudomonas aeruginosa, Gram-negative bacterium is an opportunistic pathogen and often cause infections in immunocompromised patients. It can cause both acute (pneumonia or bloodstream infections) and chronic infections such as patients with a genetic disease, cystic fibrosis, often develop chronic lung infection by *P. aeruginosa* which can last for months and decades (Grant & Hung, 2013). Multidrug resistance strains of *P. aeruginosa* and Methicillin-resistance *Staphylococcus aureus* (MRSA) are falling in the list of Centers for Disease Control and Prevention, Office of Infectious Disease Antibiotic resistance threats in the United States 2019 of antibiotic-resistant strains of bacteria and fungi. In 2017, multidrug-resistant *Pseudomonas aeruginosa* caused an estimated 32,600 infections among hospitalized patients and 2,700 estimated deaths in the United States as per CDC July 2020. The major microorganisms involved in community-acquired urinary tract infections is Uropathogenic *Escherichia coli* (UPEC), *Klebsiella pneumoniae*, and *Proteus mirabilis* (Flores-Mireles, Walker, Caparon, & Hultgren, 2015). UPEC can cause infections by several ways among which prominent is biofilm formation (Terlizzi, Gribaudo, & Maffei, 2017).

The following are bacterial species which are falling in the list of **urgent antibiotic resistance threats** by CDC, 2019:

- Carbapennem-resistance *Acinetobacter:* It is known for its various strategies to protect from antibiotics. Some strains of this multidrug resistance *Acinetobacter baumannii*, for instance, is known to produce biofilms and thus further contribute to antibiotic tolerance (Song, Cheong, Noh, & Kim, 2015).
- *Clostridioides difficile:* This is a Gram-positive sporulating anaerobic bacillus, is a causative agent of diarrhea. The formation of biofilms in *C. difficile* is not only responsible for antibiotic resistance but also the recurrence of disease (James et al., 2017).
- Carbapennem-resistance Enterobacteriaceae: In one study, it has been found that all the 10 strains of Carbapennem-resistance Enterobacteriaceae isolated produces biofilms both on microtiter biofilm and continuous flow chamber and are the cause of bloodstream infections (Yaita et al., 2019). It has also been studied that Enterobacteriaceae bacteria isolated from biofilms confers higher antibiotic resistance than planktonic bacteria (Hashem, Abd El Fadeal, & Shehata, 2017; W. S. Li, Chen, Kuo, Chen, & Lee, 2018; Qin et al., 2017).
- Drug resistance *Nisseria gonorrhoeae*: It also produces biofilms and thus contributes to antibiotic resistance, persistence, and asymptomatic infections (Steichen, Shao, Ketterer, & Apicella, 2008).

The following list of bacteria considered to be **serious antibiotic resistance threats** by CDC (2019):

- Drug-resistance *Campylobacter*: Poultry chicken is the main reservoir of Camplylobacter, the main causative agent of gastroenteritis. Zhang et al in 2017, investigated the first time the presence of Camplylobacter in chicken and studied antibiotic resistance concerning biofilm (T. Zhang et al., 2017). It has been found that biofilm-producing strains pose higher resistance to antibiotics in contrast to weak or no biofilm-producing strains. Moreover, they also found that biofilm strains are posing antibiotic resistance to many antibiotics towards which non-biofilm producing is sensitive (T. Zhang et al., 2017).
- ESBL-producing Enterobacteriaceae: Biofilm production is highly prevalent in the tested virulent clinical strains of extended-spectrum beta-lactamase (ESBL) producing *Klebsiella pneumoniae* in contrast to *E. coli* (Y. Zhang, Cao, Wang, & Xiao, 2013)

- Vancomycin-resistance *Enterococci* (VRE): A study conducted on clinical isolates of *Enterococci faecium* found that biofilm-producing strains confer more resistance than non-biofilm producing and further found that biofilm strains are more virulent and pose resistance genes (Goudarzi, Mobarez, Najar-Peerayeh, & Mirzaee, 2018).
- Drug-resistance nontyphoidal *Salmonella*: nontyphoidal *Salmonella* causes foodborne illnesses. It has been found that the clinical strains producing biofilms are also conferring higher antibiotic resistance (W. Li et al., 2017)
- Drug-resistance *Salmonella* serotype Typhi: It has also been found that biofilms are not only enhancing antibiotic tolerance but also acts as a source of cross-contamination in the food industry (Kim & Wei, 2007).
- Drug resistance *Shigella*: In a recent article related to antibiotic resistance in *Shigella*, it has clarified that biofilm-forming *Shigella* confers higher antibiotic resistance and it is a matter of concern (Ranjbar & Farahani, 2019).
- Drug-resistance *Streptococcus pneumoniae*: It is a human commensal usually colonizing nasopharynx and biofilm-forming pneumococcus is also drug-tolerant than planktonic counterparts (Chao, Marks, Pettigrew, & Hakansson, 2015)
- Drug-resistance Tuberculosis: Similar antibiotic tolerance/resistance was observed in mycobacterial biofilms (Islam, Richards, & Ojha, 2012)

Most of the biofilm-forming antibiotic-resistant bacterial strains highlighted by the CDC are conferring a higher degree of antibiotic resistance in contrast to planktonic cells and are a major concern as their treatment with existing antibiotics is diminishing. It is an alarming situation to find out new therapeutic strategies to cope up with this enhanced antibiotic resistance associated with biofilm formation.

1.1.4. Antibiotic resistance and tolerance

As it has already been covered in the previous sections that biofilms are one of the major causes of chronic infections and they are highly resistant/tolerant to antibiotics. The major question that arises is that how biofilms would succeed in making themselves more reluctant to be treated with antibiotic treatment. Before discussing the mechanisms of resistance and/or tolerance in biofilm, it would be better to understand the difference between "**resistance**" and "**tolerance**". *"**Resistant microorganisms can grow in the presence of a bactericidal or bacteriostatic antimicrobial agent at a concentration that would normally be inhibitory to growth. Resistance is typically measured in planktonic cultures using the minimum inhibitory concentration (MIC), which is the lowest concentration of antimicrobial agent that will***

inhibit the growth of the microorganism. Resistance is most often thought of as being attributable to mutations or exchange of antibiotic resistance genetic elements (acquired resistance), although resistance may also be intrinsic and thus dependent on wild-type genes and innate properties of the cell" (Hall & Mah, 2017).*"**In contrast, tolerance to an antimicrobial agent is the ability of a microorganism to survive, but neither grow nor die, in the presence of a bactericidal antimicrobial agent. A measure of tolerance is the minimum bactericidal concentration (MBC), which is the lowest concentration of a bactericidal antimicrobial that will kill ≥99.9% of cells in a culture**"*(Hall & Mah, 2017).

Many of the bacterial biofilms do not allow the antimicrobial agents to penetrate but certain compounds can move inside but still, biofilms are resistant to antibiotics (Anderl, Franklin, & Stewart, 2000; Stone, Wood, Dixon, Keyhan, & Matin, 2002). Using *Klebsiella pneumoniae* as a model organism the diffusion assay was carried out to see if the antibiotics, ampicillin, and ciprofloxacin can penetrate biofilms or not. It has been found that both the antibiotics penetrated fully into the biofilms and thus suggested that some another resistance mechanism is involved (Anderl et al., 2000). Hall and Mah in 2017, explained in their review article that components of the matrix such as polysaccharides, enzymes (that can modify antibiotics), extracellular DNA, and bacteriophages are involved in different ways and thus increasing the antibiotic tolerance and resistance of biofilms (Hall & Mah, 2017).

1.1.5. Biofilm composition and development of relevant bacterial species

One of the major differences between the biofilms and planktonic cells is the production of extracellular matrix and forming complex distinctive architecture (Sutherland, 2001). This extracellular matrix is providing protection and structure to the biofilms. The basic composition of bacterial biofilms is the same such as exopolysaccharides, proteins, eDNA (extracellular DNA), lipids, phospholipids, some nutrients, and secreted metabolites (Beloin et al., 2008). Matrix also brings cell close to each other and thus make their interactions much more convenient.

1.1.5.1. *Escherichia coli*

E. coli is a Gram-negative bacterium mostly colonizing in the intestine of animals and benefiting the host by preventing the growth of pathogenic bacteria (Sharma et al., 2016). It is facultative anaerobic rod-shaped, and non-sporulating in nature. It has been used extensively in laboratories to study various aspects of bacterial physiology and biotechnology (Blount, 2015). Some *E. coli* strains are known for their pathogenesis and can cause infections in humans and

animals (Allocati, Masulli, Alexeyev, & Di Ilio, 2013). Most of the pathogenic *E. coli* infections are associated with biofilm formation and thus makes them difficult to eradicate.

The major component of *E. coli* matrix is curli fibers and it can bind with Congo dye and produces reddish-brown color colonies (Cimdins & Simm, 2017). Curli fibers are composed of two components, CsgA (major component) and CsgB (minor component), where CsgB 'nucleate' polymerization of the fibers of CsgA (Bian & Normark, 1997). These curli fibers are arranged to form β-sheets and are categorized as 'amyloid-fibers' (MARTEN Hammar, Bian, & Normark, 1996). Homologous operon of *csgBA* is also found in *Salmonella* spp. and is known as *agfBA* (Römling, Bian, Hammar, Sierralta, & Normark, 1998). CsgA protein consists of three regions (Collinson, Parker, Hodges, & Kay, 1999):

- The Sec-signal
- 22 amino acid residue N-terminal (protease sensitive)
- 109 residue C-terminal (protease-resistant)

One additional operon csgDEFG operon encodes the positive transcriptional regulator CsgD which regulates, *csgBA,* and hence, the synthesis of curli (M. Hammar, Arnqvist, Bian, Olsén, & Normark, 1995). CsgA and CsgB are synthesized within the cell and secreted outside the cell through a pore formed by membrane-localized translocator protein, CsgG (Robinson, Ashman, Hultgren, & Chapman, 2006). CsgE is a periplasmic protein that can provide specificity for the secretion of mature curli subunits through CsgG pore (Nenninger et al., 2011). CsgF is also transported outside the cell by membrane-localized CsgG porin and is required in the nucleation of CsgA by CsgB (Chapman et al., 2002). Curli fibers of *E. coli*, have similarities with the amyloid fibers associated with the eukaryotic disease, Alzheimer (Xuan Wang, Daniel R Smith, Jonathan W Jones, & Matthew R Chapman, 2007).

The second component of biofilm is pEtN-cellulose (which is recently discovered having modified with phosphoethanolamine group) (Thongsomboon et al., 2018) and was first identified in *Salmonella* and *E coli* where the gene responsible for the synthesis are found to be conserved in both the species (Zogaj, Nimtz, Rohde, Bokranz, & Romling, 2001). Later in studies, two genetic elements were found which are involved in the synthesis of pEtN-cellulose, *bcsQABZC,* and *bcsEFG* in biofilm formation (Solano et al., 2002). BcsA and BcsB are the major components of the pEtN-cellulose synthesis unit as they both are involved in the synthesis and translocation of subunits (Omadjela et al., 2013). Omadjela and group in 2013, also found that BcsA is a catalytic active subunit but BcsB is required for catalysis of UDP-glucose to pEtN- pEtN-cellulose (Omadjela et al., 2013). Thongsomboon et al, in 2018 found

that pEtN-cellulose in *E coli* and *Salmonella* spp. is chemically modified and they did it with the use of solid-state nuclear magnetic resonance, they discovered that pEtN-cellulose in the biofilm of *E coli* is modified with phosphoethanolamine group (Thongsomboon et al., 2018).

In addition to curli and pEtN-cellulose, there is poly-β-1,6-*N*-acetyl-D-glucosamine that is being produced by *E. coli* by *pgaABCD* operon and mediates cell-to-cell and cell-to-surface adhesion in biofilms (Xin Wang, Preston, & Romeo, 2004). PgaC is an apparent glycosyltransferase that is required for PGA synthesis (Itoh et al., 2008). PgaA contains a predicted β-barrel porin and forms the outer membrane secretin for PGA and PgaB contains predicted carbohydrate-binding and polysaccharide *N*-deacetylase domains (Itoh et al., 2008). This polymer was first identified in *Staphylococcus epidermidis* and referred to as intracellular adhesions (Mack et al., 1996). Later similar structured have also been found in *Staphylococcus aureus* (McKenney et al., 1999).

1.1.5.2. Enteroaggregative *E. coli* (EAEC)

The most recently identified diarrheagenic *E. coli* is the enteroaggregative *E. coli*. It can cause persistent diarrhea and malnutrition in children and HIV-infected persons and is the second most common cause of traveler's diarrhea (Kaur, Chakraborti, & Asea, 2010). EAEC strains are relatively heterogeneous and have complex pathogenesis. Pathogenesis of EAEC begins with adherence of bacteria to the intestinal mucosa by aggregative adherence fimbriae and adherence factors followed with encrusting of EAEC on the surface of enterocytes with the help of increased mucous production and finally the bacteria releases toxins and thus, leads to the elicitation of an inflammatory response, mucosal toxicity and intestinal secretion (Harrington, Strauman, Abe, & Nataro, 2005; Nataro, 2005).

During the developmental stages of pathogenesis, EAEC produces thick aggregating biofilm once adheres to the mucosa (Wakimoto et al., 2004). Biofilm forming ability of some porotypes of EAEC requires aggregative adherence fimbriae but many others form equally comparable biofilms without expressing aggregative adherence fimbriae (Sheikh, Hicks, Dall'Agnol, Phillips, & Nataro, 2001). The production of biofilm is being also regulated by AggR and requires several genes such as Fis, which encodes for a DNA-binding protein involved in growth regulation and YafK gene, which is required for transcription of aggregative adherence fimbriae/II-encoding genes (Kaur et al., 2010; Sheikh et al., 2001). Other genes that are known to be involved in biofilm formation of EAEC are EilA (HilA-like regulator) and *air* (encoding a predicted OMP in EAEC) (Kaur et al., 2010). It has also been observed that loss of biofilm

formation and diffused adherence pattern was observed in acidic environment and under neutral pH at around 7.4 typical aggregative adherence pattern was observed (Kaur et al., 2010).

1.1.5.3. Uropathogenic *E. coli* (UPEC)

Urinary tract infections are widespread infections that is affecting about 150 million people all over the world each year with high social cost (Terlizzi et al., 2017). The main cause of community-acquired urinary tract infections is the Uropathogenic *E. coli* (Flores-Mireles et al., 2015; Foxman, 2014). Other microbes that are associated with urinary tract infections are *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Streptococcus bovis*, and the fungus *Candida albicans* (Terlizzi et al., 2017). UPEC colonizes the urinary bladder with the help of various virulence factors such as lipopolysaccharides (LPS), polysaccharide capsule, flagella, outer-membrane vesicles, pilli, curli, non-pilus adhesins, outer-membrane proteins (OMPs), as well as secreted toxins, secretion systems, and TonB-dependent iron-uptake receptors, including siderophore receptors (Terlizzi et al., 2017). Among all these factors curli fibrils are the matrix component of biofilms in the case of *E. coli* and it is coordinated by the same set genes (operon *csgDEFG*) and facilitate the secretion of CsgA where CsgB nucleates CsgA subunits into curli fibers (Chapman et al., 2002; Evans & Chapman, 2014). Most recently it has been reported that flagella play an important role in adherence, maturation, and dispersal (Nakamura et al., 2016)

1.1.5.4. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative biofilm bacterium that is one of the most virulent human opportunistic pathogens and can cause a variety of acute infections such as Ventilator-associated pneumonia (Chastre & Fagon, 2002). Because of its biofilm nature, *P. aeruginosa* can colonize on catheters, indwelling devices, etc (Cole, Records, Orr, Linden, & Lee, 2014). It co-colonizes in lung tissues of the patients suffering from cystic fibrosis and thus leading to more complications and thus increases the death rate (Sadikot, Blackwell, Christman, & Prince, 2005). The antibiotic resistance of *P. aeruginosa* is remarkable and thus making it difficult to eradicate (Pang, Raudonis, Glick, Lin, & Cheng, 2019).

The biofilm components of *P. aeruginosa* includes the following components:

- Exopolysaccharides Psl: It is a galactose-rich and mannose-rich exopolysaccharide which is under the control of *psl* gene cluster containing 15 co-transcribed genes (Ma, Lu, Sprinkle, Parsek, & Wozniak, 2007)

- Exopolysaccharide Pel: It is positively charged exopolysaccharide containing two subunits, N-acetylgalactosamine and N-acteylglucosamine partially acetylated with 1-4 glycosidic linkages (Jennings et al., 2015).
- Alginate: It is synthesized in the cytoplasm as a linear polymer of D-mannuronic acid which gets modified during the process of translocation. It gets selectively O-acetylated in the periplasm by a set of genes as AlgI, AlgJ, and AlgF (Franklin, Nivens, Weadge, & Howell, 2011).
- Extracellular DNA (eDNA): It is a structural component of the biofilm matrix and it has been investigated that it acidifies the environment of biofilm and planktonic cultures thus pose higher resistance to aminoglycoside (Wilton, Charron-Mazenod, Moore, & Lewenza, 2015).
- Proteins: Proteins are progressively produced during the development or maturation of biofilm. Proteins associated with antibiotic resistance and virulence are continuously increasing with the development of biofilm. There are several proteins which are specifically controlling the developmental stages of biofilm maturation in *P. aeruginosa* (Southey-Pillig, Davies, & Sauer, 2005).

Biofilm synthesis in *P. aeruginosa* is controlled by various factors such as c-di-GMP (a ubiquitous intracellular second messenger molecule), GacA/GacS two-component system, quorum sensing, etc. (Wei & Ma, 2013).

1.1.5.5. *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive human commensal that is also one of the major human pathogens that can cause skin infections to life-threatening sepsis. It is directly associated with implant-associated infections (Costerton, Stewart, & Greenberg, 1999). The biofilm matrix of *S. aureus* is composed of polysaccharides, eDNA, proteins, and many other host factors and the quantity of each component depends on environmental conditions (Abraham & Jefferson, 2012; Lister & Horswill, 2014). The major component of their biofilms is β-1,6-linked N-acetylglucosamine polymer (PNAG) which is also known as polysaccharide intercellular adhesins (PIA) (O'Gara, 2007) which also pose resistant to phagocytosis (Brooks & Jefferson, 2014). PNAG is produced and exported by proteins expressed by *icaADBC* locus. *S. aureus* also produces icaADBC independent biofilms which are being mediated by many surface-associated proteins such as protein A (Merino et al., 2009), fibronectin-binding proteins (O'Neill et al., 2008), surface protein (SasG), clumping factor B (Abraham & Jefferson, 2012), Aap and Biofilm associated protein (Bap) (Brooks & Jefferson, 2014; Cucarella et al., 2001).

Sub-population of *S. aureus* can undergo suicide using autolytic enzymes synthesized by *atl* and *lytM* genes and thus releasing extracellular DNA (eDNA) (Thomas & Hancock, 2009). It has also been identified that eDNA plays an important role in *S. aureus* in attachment and forms an integral part of the biofilm matrix (Mann et al., 2009).

1.1.5.6. *Bacillus subtilis*

Bacillus subtilis is a Gram-positive, non-pathogenic endospore-forming soil bacterium. The cells are short and motile and they form long chains of non-motile cells, the moment they adhere to the surface develops biofilm and secrets extracellular matrix (Vlamakis, Chai, Beauregard, Losick, & Kolter, 2013). It produces wrinkled colonies on solid medium and in liquid, they either produce pellicle over the air-liquid interface or just float on the surface of the liquid (Vlamakis et al., 2013). *epsA-epsO* operon synthesis the major exopolysaccharide component in all types of biofilms. EpsE of *epsA-epsO* operon is the major protein that is involved in the production of EPS and it also inhibits the motility (Blair, Turner, Winkelman, Berg, & Kearns, 2008). γ-poly-DL-glutamic acid (PGA) is another exopolymeric substance produced by *B. subtilis* which can also enhance submerged biofilm formation (Morikawa et al., 2006). This component is not associated with the solid surface associated with biofilms and pellicle (Branda, Chu, Kearns, Losick, & Kolter, 2006).

In addition to exopolysaccharides, some structural proteins are associated with a biofilm of *B. subtilis,* Tas A and BslA. TasA is the major protein component which is and in the absence of this *B. subtilis* cannot form a 3D biofilm structure (Branda et al., 2006). TapA (TasA anchoring/assembly protein) is a cell wall-associated protein that is not only anchoring the TasA amyloid-like fibers but is also involved in the assembly of TasA components (Romero, Vlamakis, Losick, & Kolter, 2011). BslA is another major protein that is hydrophobic and resists gas penetration (Kobayashi & Iwano, 2012). As it forms a water-resistant layer on top of the biofilm so it is termed as biofilm surface layer protein (BslA) (Kobayashi & Iwano, 2012).

1.2. Molecular network controlling *E. coli* biofilms

Before discussing molecular networking, it is important to understand and answer the biggest questions such as when, how, and why bacteria tend to produce extracellular matrix? Under nutrient deficiency, and/or stressed conditions, bacteria have to change their strategy to control the death rate and at a cellular level, it means they have to shift from growth to survival mode. To achieve a survival strategy, they produce energy extensive extracellular matrix for protection

and to build homeostasis as the extracellular space within a biofilm is at the same order of magnitude as the intracellular volume of the cells (R. Hengge, 2020).

The components of biofilms production are under the control of complex molecular networking systems which regulates the switch between planktonic and biofilm formations or in other words between growth and survival. Using *E. coli* as a model bacterial, this review will highlight the number of factors that decide the lifestyle of bacteria. Generally, microorganisms can sense changes in their vicinity such as stress caused due to lack of nutrients, osmolarity, oxygen depletion or host-derived signals, sub-inhibitory concentrations of antibiotics, etc., and in response to that, bacteria tend to form biofilms and also show physiological heterogeneity within the biofilm in response to changes in their microenvironment (Hoffman et al., 2005; Karatan & Watnick, 2009; D. O. Serra & Hengge, 2014). The following factors are involved in the overall trade-off between growth and maintenance/resilience.

1.2.1. Sigma factors

RNAP (RNA polymerase) core enzyme is at the top of the hierarchical control unit for which the major sigma factors are competing to control the switch between growth and survival (Browning & Busby, 2016). Following are the sigma factors of RNAP which are known in *E. coli* and play a significant role under different conditions:

- RpoD (σ^{70}): It is the vegetative sigma factor which is controlling all the "house-keeping" genes in growing cells (Weber, Polen, Heuveling, Wendisch, & Hengge, 2005).
- RpoS (σ^{S}): It is another vegetative sigma factor which is controlling the genes required to cope with the stress and non-optimal growth conditions. So, it is usually considered a master regulator of general stress response in *E. coli* (Weber et al., 2005).
- σ^{FliA} (σ^{28} or RpoF): It is an alternative sigma factor that is involved in the synthesis of enzymes and proteins which are needed for the formation of flagella at the post exponential phase of bacterial growth along with RpoD (D. O. Serra & Hengge, 2014).
- Other transient sigma factors: This includes σ^{32} (RpoH), σ^{E} (σ^{24} or RpoE), and σ^{54} (RpoN) which are controlling the expression of genes required for dealing with heat shocks, extra-cytoplasmic stress, and nitrogen limitations respectively (D. O. Serra & Hengge, 2014).

Under stressful situations, RpoS manages to more or less take over the core RNAP at the expense of RpoD activity and therefore growth.

1.2.2. Transcription factor cascades

The backbone of the regulatory system is the transcription factor cascade which works at different levels. CRP is one such control unit in *E.coli* that organizes growth and metabolism in the presence of limited resources (Busby & Ebright, 1999; You et al., 2013). Two other important transcriptional factors that at lower level works inversely to control the switch between flagellated cells and adhesive multicellularity are FlhDC and CsgD (Chevance & Hughes, 2008; Pesavento et al., 2008). These transcription factors control many other genes (R. Hengge, 2020).

FlhDC is the master operon that encodes the transcription factors FlhD and FlhC that begins the intricately programmed process of flagellar synthesis in *E. coli* (Fahrner & Berg, 2015). The heterohexameric complex ($FlhD_4C_2$) of homodimers of FlhD and FlhC activates the transcription of various RpoD dependent genes including genes required for synthesis and regulation of flagellar apparatus (Fahrner & Berg, 2015). It also regulates FliA sigma factor that is required for the gene involved in motility and chemotaxis (Fahrner & Berg, 2015). FlhDC/ FliA sigma factor also drives expression of PdeH (master phosphodiesterase), GTPase signaling (RdcA/RdcB), and c-di-GMP binding effector protein, YcgR (R. Hengge, 2020).

The FixJ/LuxR family protein CsgD is a key transcriptional factor for both curli and pEtN-cellulose. CsgD is directly regulating *csg* operon, which is involved in the synthesis, translocation, and assembly of amyloid fibers (M. Hammar et al., 1995). It is also controlling the synthesis of the second messenger, ci-di-GMP by regulating the gene expression of diguanylate cyclase enzyme, DgcC, which is further involved in the regulation of the synthesis of pEtN-cellulose (Richter et al., 2020). Because of the involvement of several transcription factors for the regulation of *csgD* promoter, including CpxR, Crl, H-NS, IHF, OmpR, RstA, MlrA, RcsB and CRP, *csgD* is considered as one of the most complex promoters in *E. coli* (Ogasawara, Yamamoto, & Ishihama, 2010). MlrA is a MerR-like regulator which is expressed at the stationary phase as both RpoD and RpoS are involved in the transcription initiation of the mlrA gene in *E. coli* and *Salmonella* (Brown et al., 2001). It was investigated that it is the novel positive regulator for the synthesis of extracellular matrix and curli fimbriae (Brown et al., 2001). MlrA is a DNA-binding regulatory protein as it contains a conserved N-terminal DNA-binding domain (Ogasawara, Yamamoto, et al., 2010). MlrA is the target of the complex c-di-GMP regulation by PdeH/DgcE/PdeR/DgcM to control the regulation of *csgD* promoter (which

is well explained in the following section) and finally controlling the major components of biofilm formation .

1.2.3. Small Signaling molecules

The switch between different states of bacteria requires triggers and drivers that generally provided by nucleotides for instance the concentration the nutritional conditions is reflected by GTP and (p)ppGpp, cAMP, or c-di-GMP are second messenger molecules that are enzymatically synthesized or degraded in response to specific environmental or cellular stimuli (R. Hengge, 2020). In *E. coli*, cAMP synthesis and degradation are under the control of single adenylate cyclase (Cya) and single specific phosphodiesterase (CpdA) respectively (Galperin, 2018; Matange, 2015) and generally activated in the late exponential phase of growth (You et al., 2013). CRP is the major transcriptional hub of cAMP that can directly or indirectly regulate many target genes (Grainger, Hurd, Harrison, Holdstock, & Busby, 2005).

Flagellar operon, *flhDC* is the most interesting target of cAMP and thus enabling the cells to produce more flagella with enhanced motility representing the foraging strategy when under nutrient-deficient conditions (Barembruch & Hengge, 2007; K. Zhao, Liu, & Burgess, 2007). The role of the cAMP is to downregulate the RpoS response by broadly expanding the utilization of nutrient responses, conversely, (p)ppGpp is reducing the cellular growth in response to decreasing nutrient availability and promotes the RpoS accumulation (Potrykus, Murphy, Philippe, & Cashel, 2011; Zhu, Pan, & Dai, 2019). RelA (ribosome-associated synthase activated at the time of amino acid starvation) and SpoT (bifunctional enzyme; either antagonize RelA by degrading (p)ppGpp or synthesize (p)ppGpp) are maintaining the cellular level of (p)ppGpp (R. Hengge, 2020). SpoT can interact with acyl carrier proteins (ACP) during the decreasing fatty acid synthesis which is a signal for the starvation of carbon source and thus activates the synthesis of (p)ppGpp (Angelini, My, & Bouveret, 2012; Battesti & Bouveret, 2006). The cAMP is managing growth under nutrient-deficient situations and (p)ppGpp adjusts the survival of the cell under growth-reducing resource limitation and stresses. (p)ppGpp promotes RpoS accumulation and activity and induces multiple stress responses (R. Hengge, 2020).

The expression of the major components of biofilm, amyloid fibers, and pEtN-cellulose in *E. coli* are being regulated by the RpoS stress response regulator via a major transcriptional factors CsgD (R. Hengge, 2020). There is a network of 12 diguanylate cyclases and 13 specific phosphodiesterases that make and break c-di-GMP, another second messenger molecule that plays important role in controlling the whole cascade of biofilm formation (Regine Hengge,

2009). This molecule is a key component between growth, flagellar activity, motility and multicellularity lifestyle by inversely coordinating cAMP/CRP/FlhDC/ σ^{FliA} and RpoS/CsgD (Lindenberg, Klauck, Pesavento, Klauck, & Hengge, 2013; Pesavento et al., 2008). Almost all the diguanylate cyclases and phosphodiesterases express parallel and modulate the concentration of c-di-GMP level which overall control distinct targets such as flagellar rotations, transcription of *csgD, the* activity of cellulose synthase (R. Hengge, 2020). The higher concentration of c-di-GMP can trigger biofilm formation in cells and plays an important role in driving the bacterial planktonic cells to a sedentary lifestyle of biofilm formation (Valentini & Filloux, 2016).

The master PDE is the PdeH in *E. coli* that maintains the c-di-GMP to the lowest cellular level and that goes up at the entry of stationary phase by top-level DGC, DgcE that is regulated by RpoS and activated by RcdA/RcdB (the sensory system for the decreasing concentration of GTP) (Pfiffer, Sarenko, Possling, & Hengge, 2019; Sarenko et al., 2017). With increasing cellular levels of c-di-GMP, the flagellar rotation gets inhibited upon the binding of c-di-GMP withYcgR and thus tunes down the motility at the entry of the stationary phase (R. Hengge, 2020). In addition to this, spiking levels of c-di-GMP also provide positive input in the expression level of biofilm formation master regulator CsgD by controlling the activity of MlrA (R. Hengge, 2020).

Besides having c-di-GMP synthesizing activity, DgcM can also activate MlrA by directly interacting with its C-terminal ligand domain (Lindenberg et al., 2013). This interaction is inhibited by PdeR, but with the increasing concentration of c-di-GMP with DgcE and DgcQ, PdeR will no longer be available to be a barrier between the interaction of DgcM with MlrA and thus release and activates MlrA (Klauck, Serra, Possling, & Hengge, 2018). This activated form of MlrA binds to the upstream region near to another activator OmpA site on the *csgD* promoter and thus initiates the transcription of *csgD* (Ogasawara, Yamamoto, et al., 2010). DgcM is also positive feedback of the entire regulatory network between DgcM, MlrA, and PdeR to activate the CsgD as it keeps PdeR busy by increasing the level of c-di-GMP (R. Hengge, 2020).

At the lowest level of the hierarchy, DgcC is specifically activating the BcsA and BcsE, membrane-located cellulose synthase complex and modification respectively, and several phosphodiesterases act here (R. Hengge, 2020). Overall, cellular levels of c-di-GMP play a significant role in balancing the growth and survival strategy.

1.2.4. Small regulatory mRNA and stress responses

Small regulatory RNAs are fine-tuning and interconnecting the expression of RpoS, FlhDC, and CsgD with each other and some additional stress response systems by providing signal inputs at the mRNA levels (R. Hengge, 2020). Multiple environmental stresses are sensed by *E coli* in highly sophisticated and complex networking of various protein molecules to finally regulate biofilm formation via CsgD (Mika & Hengge, 2014). OmpR and CpxR are activators and repressors of *csgD* respectively having overlapping recognition sites upstream the RNS polymerase binding site and works competitively in a changed osmolarity environment (Jubelin et al., 2005). The same study also revealed that H-NS (histone-like nucleoid structuring protein) mediates repression of *csgD* in high glucose medium. H-NS can upregulate or downregulate *csgD* under different environmental osmolarity conditions (Jubelin et al., 2005).

Another environmental cue sensed by *E. coli* is a phosphorelay cascade that is involved in biofilm formation is Rcs phosphorelay. RcsC, sensor kinase, senses environmental stimuli and gets autophosphorylated which then transfers phosphate to the response regulator, RcsB which then regulates the number of genes (Clarke, 2010). When Rcs phosphorelay pathway is active (phosphorylated), or RcsB is a phosphorylated sRNA molecule, RprA gets accumulated and thus represses the biofilm synthesis by inhibiting the transcription of *csgD* mRNA (Latasa et al., 2012). RpoE is another environmental stress regulator which was also identified as one associated with biofilm formation in *Salmonella pullorum* (Huang et al., 2015). Additional fine-tuning of CsgD is done by a set of sRNA molecules (Mika & Hengge, 2014). There are nearly seven known sRNA molecules like RprA, McaS, OmrA/omrB, RydC, GcvB, RybB that are downregulating the expression of CsgD by directly interacting with the mRNA molecule of *csgD* (Andreassen et al., 2018; Mika & Hengge, 2014).

In summary, biofilm synthesis is regulated by a complex networking system that works in a highly controlled and sophisticated manner. The c-di-GMP is a key switch between planktonic and biofilm formation in the case of *E. coli* and many other bacteria. This key switch works efficiently with the contribution of many sigma factors of RNAPs which are controlling the expression level of enzymes required for the synthesis and degradation of the c-di-GMP and thus tune the concentration as per the requirement of the situation. In totality, all the molecules, c-di-GMP, RNAPs, transcriptional regulators, and sRNA molecules work in close association with each other to change the lifestyle of bacterial from motile to biofilm.

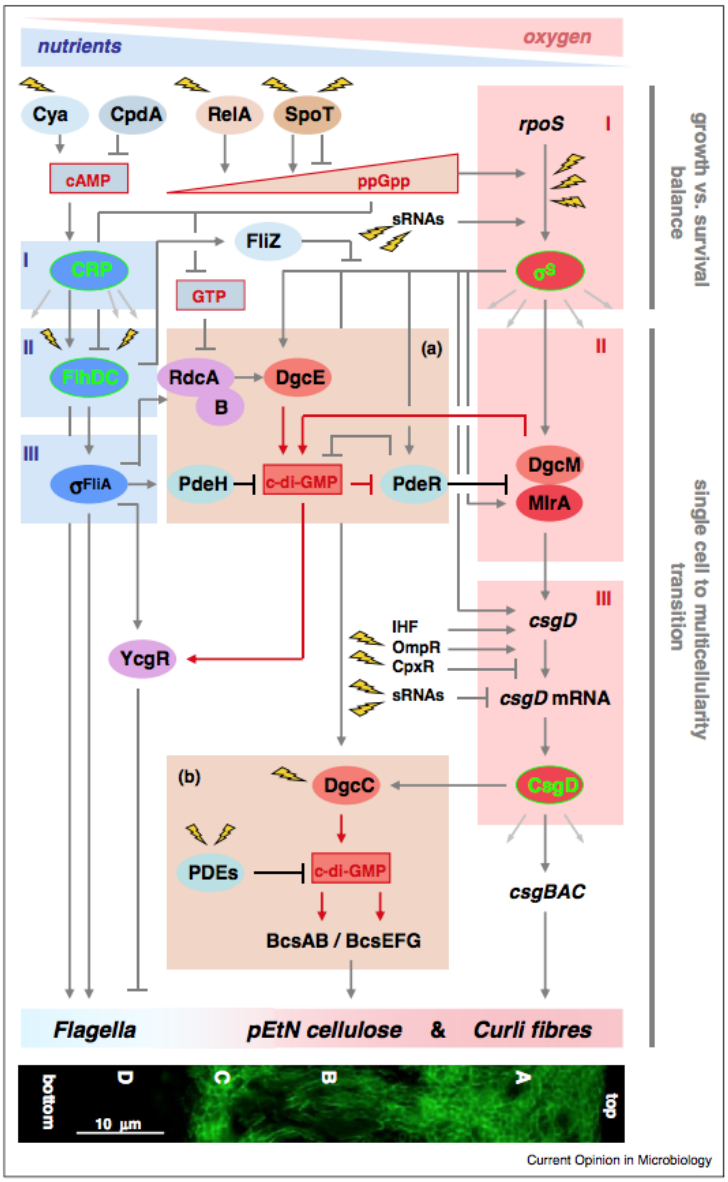


Figure 1: Regulatory molecules controlling and balancing the growth and survival in *E. coli* (figure adapted from (R. Hengge, 2020)). The major transcriptional factors are texted in green color. Signaling and regulatory proteins are shown by ovoid symbols (blue color component indicates flagellar cascade, red color shows the components involved in biofilm formation, and purple signifies the flagellar controlling cascade). At the bottom, vertical cryosection of the macrocolony of *E. coli* (major components of the matrix, curli, and pEtN-cellulose were stained with fluorescent dye Thioflavin-S)is shown and the image is rotated to 90 deg. C. to roughly correlate the activities in the section with the regulatory components. This figure has been used here with permission (R. Hengge, 2020)

1.3. Current strategies to target biofilms

As the review already covered that several infections are highly making the situations of the patients worse because the causative agent can undergo biofilm formation and thus makes the treatment difficult with the current antibiotic techniques. Due to the lack of effective treatment

antibiotics so, it is urgent to search for alternative and novel strategies to cope up with biofilm formers.

1.3.1. Strategies based on plant-derived anti-biofilm agents

Natural resources such as plant material or compounds of plant origin are extensively studied in search of new treatment techniques to control bacterial growth in the host (Cowan, 1999). So, most recently many plant extracts are being explored by many researchers to find a solution to deal with biofilm formation (Coenye et al., 2012). Coenye et al investigated, 119 plant extracts and found only 5 (*Epimedium brevicornum, Malus pumila, Polygonum cuspidatum, Rhodiola crenulata,* and *Dolichos lablab*) showing potent activity against *Propionibacterium acnes* and they also identified the active compounds in it i.e. icariin, resveratrol, and salidroside (Coenye et al., 2012). In 2012, alcoholic extract of *Melia dubia* was found to be active against haemolysis, swarming motility, hydrophobicity, and biofilm formation (Ravichandiran, Shanmugam, Anupama, Thomas, & Princy, 2012). *Capparis spinose* showed strong anti-quorum sensing and antibiofilm activity at 0.5-2 mg/mL concentration (Issac Abraham, Palani, Ramaswamy, Shunmugiah, & Arumugam, 2011). They used specific quorum sensing and biofilm-forming dependent phenotypic characteristics of a set of bacterial strains such as *Chromobacterium violaceum*, *Pseudomonas aeruginosa* PAO1, *E. coli, Proteus mirabilis,* and *Serratia marcescens*, to analyze the quorum sensing and biofilm activity (Issac Abraham et al., 2011).

In 2016, Serra et al demonstrated that epigallocatechin gallate (EGCG), a polyphenolic compound found in green tea inhibits the biofilm formation by targeting the curli subunits in the biofilm of *E. coli* (D. O. Serra, Mika, Richter, & Hengge, 2016). *Azadirachta indica* (Neem) is the most efficient in reducing and removing biofilms of *Mycobacterium smegmatis* (Abidi, Ahmed, Sherwani, Bibi, & Kazmi, 2014). Abidi with his co-workers also found that extracts of *Vaccinium oxycoccos* (Cranberry) have strong antibacterial activity against *M. smegmatis* (Abidi et al., 2014). Transcriptional analysis of an extract of *Carex dimorpholepis* showed to reduce the expression of curli genes, motility genes, and AI-2 quorum sensing genes in enterohemorrhagic *E. coli* (EHEC) (J. H. Lee et al., 2013). Ethanolic extract of *piper betle* (Thai traditional herb) found to be effective against biofilm. It has the potential to prevent and eradicate biofilm formation and the most active compound in it is 4-chromanol (Teanpaisan, Kawsud, Pahumunto, & Puripattanavong, 2017). Extracts of sage leaf, purple coneflower flower, and licorice root found to be effective against planktonic growth and biofilm formation of *Streptococcus pyogenes* (Wijesundara & Rupasinghe, 2019).

Manuka honey has been proven to be effective against biofilm development and disassembles the already established *Streptococcus pyogenes* and this they likely to do by interrupting the binding of the *Streptococcus pyogenes* to host tissues protein fibronectin (Maddocks, Lopez, Rowlands, & Cooper, 2012). They also found that the expression level of major genes encoding fibronectin-binding streptococcal surface proteins also gets reduced with Manuka honey (Maddocks et al., 2012). In 2019, it has been shown that honey present at relatively low concentrations can inhibit the biofilm formation and can also significantly eliminated the biofilm formation of *P. aeruginosa* (J. Lu et al., 2019). It has also been investigated by the same research group that the cells recovered from biofilms treated with sub-inhibitory concentrations of honey showed reduced susceptibility to honey (J. Lu et al., 2019). They also concluded that manuka-type honeys can completely remove the already established biofilms (J. Lu et al., 2019). Low concentrations of honey is also proven to be effective against quorum sensing, biofilm, and virulence in the case of enterohemorrhagic *Escherichia coli* O157:H7 without inhibiting the growth of planktonic cells (J. H. Lee et al., 2011). The latter group also showed with their transcriptomic analysis that honey significantly repressed curli genes, quorum sensing genes, and virulence genes (J. H. Lee et al., 2011). Antimicrobial and anti-biofilm activity of four honeys i.e. *Melipona beecheii* honey (Cuba) and three *Apis mellifera* honeys from New Zealand, Cuba, and Kenya were tested against 52 clinical isolates (includes both Gram positive and Gram negative bacteria and *Candida albicans*). Overall, *M. beecheii* honey had the highest antimicrobial and antibiofilm activity (both inhibiting the biofilm formation and eradicating the already formed biofilm) (Morroni et al., 2018).

Essential oils are also natural volatile substances derived from plants. Essential oils are also been widely used because of their antimicrobial activities for a long time (Hammer, Carson, & Riley, 1999). It has been shown that thyme oil can inhibit the development of biofilm in the strains of genera *Acinetobacter, Sphingomonas,* and *Stenotrophomonas* (Sadekuzzaman, Yang, Mizan, & Ha, 2015; Szczepanski & Lipski, 2014).

These observations from different research papers can be concluded as plant extracts or products derived from plants or other natural products can be used as potential targets against biofilm formation. Lots of research is required to understand their molecular mechanisms and their relevance at the clinical level. Specifically, in nature, bacterial biofilms are mostly multispecies and thus their effects and interactions *in vivo* are still needed to be uncovered.

1.3.2. Bacteriophages as a tool to kill bacteria in biofilms

In 2011, Pires et al tested phages as a tool to control biofilm formation against biofilms of *P. aeruginosa* and they selected two phages to test against 24 hr biofilm and found that after incubation of 24 hrs, *P. aeruginosa* acquired resistance against phage, philBB-PAP21 but phage philB-PAA2 continued to destroy cells within the biofilm (Pires, Sillankorva, Faustino, & Azeredo, 2011). In the same year, Meng et al also found similar results with bacteriophage lysin, LySMP, and tested lysin alone and in combination with antibiotics and bacteriophage against *Sterptococcus suis* and found that lysin itself has the potential to disrupt the established biofilm and inactivate the cells but in combination with antibiotics, the effect of dispersal and inactivation of cells gets increased significantly (Meng et al., 2011). In the year 2007, Lu et al engineered a bacteriophage capable of expressing biofilm-degrading enzyme and found that the cell count of bacterial biofilm remarkably reduced by approximately 4.5 orders of magnitude and removed 99.997% cells in comparison to the non-engineered phage (T. K. Lu & Collins, 2007). Treating the mixed culture biofilm with bacteriophage gave a new viewpoint to study and use this technology in the real world (Milho et al., 2019). As per the study conducted by Milho with his co-workers, it has been found that treating the dual bacterial biofilm culture is not as efficient as a single species biofilm (Milho et al., 2019). Though bacteriophages look promising strategy against biofilm formation yet there are lots of drawbacks that are associated with it such as the development of phage resistance by bacteria, transduction of antibiotic resistance genes, and virulence genes by phages. With the help of synthetic biology, and proper insight of phage study in relation to bacteria and host could become an effective anti-biofilm agent to treat anti-biofilm associated infections.

1.3.3. Quorum sensing inhibitors

Quorum sensing is a phenomenon in which bacterial cells communicate with each other using chemical signals. They release, sense, and respond to small diffusible signal molecules and thus express different types of phenotypes such as bioluminescence, sporulation, competence, antibiotic production, biofilm formation, and virulence factor (Rutherford & Bassler, 2012). In contrast to this, the process of quorum sensing inhibition is termed as quorum quenching (Turan & Engin, 2018). Quorum sensing has not been covered in this review but it an important phenomenon regulating biofilm formation in many bacterial species. Biofilm inhibition by quorum quenching could be a potential way to cope up with bacterial biofilms. (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone (furanone) of the macroalga *Delisea pulchra* proved to be an effective quorum quenching molecule and inhibited biofilm formation and

swarming in *E. coli* (D. Ren, Sims, & Wood, 2001). Extract of garlic indicates quorum quenching activity and makes the *P. aeruginosa* sensitive to tobramycin and phagocytosis by polymorphonuclear leucocytes (Bjarnsholt et al., 2005). *Mangifera indica* L. (mango) has been shown to possess quorum quenching activity and was found to reduce biofilm formation by *P. aeruginosa* PAO1 and *Aeromonas hydrophila* WAF38 remarkably (Husain et al., 2017). Damte et al screened 97 plant extracts from Korea for their quorum sensing inhibition activity using 2 biosensor strains *Chromobacterium violaceum* (CV12472) and *Pseudomonas aeruginosa* (PAO1) and found that 18 in total has quorum quenching activity (Damte, Gebru, Lee, Suh, & Park, 2013).

As per studies conducted by various researchers, it can be concluded that quorum quenching agent in combination with antibiotic treatment or any other strategy can be proven to be effective for future treatment of chronic infections.

1.3.4. Nanoparticles

Nanotechnology is an emerging technology that deals with nano-meter size particles (Salata, 2004). It is a leading technology in the field of biology and medicine and also has commercial importance (Salata, 2004). Nanoparticles are the particles with overall dimensions in nanoscale less than 100 nm. They are advantageous over the bulk material by the virtue of their size (Murthy, 2007). Biologically synthesized (with *Bacillus licheniformis*) silver nanoparticles were effectively used against *P. aeruginosa* and *S. epidermidis* strains (Kalishwaralal, BarathManiKanth, Pandian, Deepak, & Gurunathan, 2010; Sadekuzzaman et al., 2015). They found that 95% of biofilm inhibition within 24 hrs of incubation and proved that there could be some future to deal with biofilm using these emerging techniques (Kalishwaralal et al., 2010). Most recently, Khan et al coupled gold nanoparticles with chitosan oligosaccharide which proved to be highly effective against biofilms of *P. aeruginosa* and proved that coupling of nanoparticles with natural compounds could be a potential agent to exhibit anti-biofilm inhibitions (F. Khan et al., 2019). Most interestingly, Gondil and his co-workers, evaluated antibacterial, anti-biofilm and biocompatible potential of green synthesized Seabuckthorn silver nanoparticles (SBT@AgNPs) against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, and methicillin-resistant *Staphylococcus aureus* and found that it efficiently eradicating young and mature biofilm of *P. aeruginosa* (Gondil, Kalaiyarasan, Bharti, & Chhibber, 2019). In addition to this, they also tested the toxicity test of SBT@AgNPs on RBCs and human dermal fibroblasts and found that it is non-toxic and does not have any membrane damage and morphology variation on RBC's and human dermal fibroblast

respectively (Gondil et al., 2019). Hybrid nano-formulation of root extracts of *Vetiveria zizanioides* with silver nanoparticles (AgNPs) showed that it is an ideal quorum quenching agent and biofilm agent against *Serratia marcescens* (Ravindran et al., 2018).

1.3.5. Enzymatic degradation of matrix

The matrix of biofilm is composed of various biomolecules like proteins, lipids, DNA, polysaccharides which are forming a structural component of the biofilm (Beloin et al., 2008). Enzymes can be used as a tool for the dispersal of the biofilm (Kaplan, 2010). Extracellular DNA is known to be an integral part of biofilm (Whitchurch, Tolker-Nielsen, Ragas, & Mattick, 2002) and can be targeted with DNAase enzyme. Treatment of antibiotics in combination with DNase enzymes revealed interesting results in the inhibition of biofilm formation as it enhanced the effect of antibiotic treatment (Tetz, Artemenko, & Tetz, 2009). Human recombinant DNAase enzyme showed remarkable results against biofilms on 96 well plates against *S. aureus* and *S. epidermidis* and it also increased the sensitivity against antibiotics at clinically achievable concentrations (Kaplan et al., 2012). A combination of nanoparticles with silver sulfadiazine (frontline therapy in burn wound infections), chitosan gel loaded with solid lipid nanoparticles of silver sulfadiazine (SSD-LSNs), along with DNAase enzyme found to be highly effective in wound healing in 21 days in comparison with marketed formulations silver sulfadiazine and SSD-LSNs (K. K. Patel et al., 2019).

Glycylglycine endopeptidase, lysostaphin cleaves pentaglycine cross-bridges in the peptidoglycan of staphylococcal and it can kill *S. aureus* within mins. This enzyme can also kill the bacteria of *S. aureus* (sensitive to lysostaphin) within biofilms and also disrupt the matrix *in vitro* whereas, biofilms of resistant strains against lysostaphin remained unaffected. Similarly, commercially alpha-amylase can also be used to inhibit the biofilm formation and dissociate the cell aggregates in liquid culture against *S. aureus* (Craigen, Dashiff, & Kadouri, 2011).

1.3.6. Biosurfactants

Biosurfactants are molecules produced by microorganisms having an ability to reduce surface tension between two insoluble phases (Otzen, 2017). Biosurfactants (obtained from *Pseudomonas aeruginosa*, *Bacillus metylotrophicus* and *Candida bombicola*) in combination with chitosan (extracted from fungus Mucorales) or sodium fluoride were used to formulate 6 combinations of toothpaste and tested against biofilms of *S. mutans* in artificially modified saliva for 24 h at 37 °C in anaerobiosis (Resende et al., 2019). It has been found that

combinations of biosurfactants obtained from *Candida bombicola* and *Pseudomonas aeruginosa* with chitosan had an additive inhibition effect against biofilms of *S. mutans* (Resende et al., 2019). Biosurfactants obtained from *Bacillus subtilis* VSG4 and *Bacillus licheniformis* VS16 was investigated for various physiological functions and it has been found that both biosurfactants showed remarkable anti-biofilm activities with the disintegration of already established biofilm with a percentage ranging from 63.9 to 80.03% for VSG4 biosurfactant, and from 61.1-68.4% for VS16 biosurfactant (Giri, Ryu, Sukumaran, & Park, 2019). In 2013, Quinn and co-workers found that the use of biosurfactant proved to be efficient against biofilms in a mixed culture which otherwise is challenging and difficult to deal with (Quinn, Maloy, Banat, & Banat, 2013). They did a comparative study between two biosurfactants (rhamnolipids and a plant-derived surfactant) and broad-spectrum antibiotics and found that biosurfactants inhibited complex heterogeneous marine biofilms in contrast to antibiotic treatment which otherwise was proven to be ineffective on mixed biofilms (Quinn et al., 2013).

1.3.7. Bacteriocins

Bacteriocins are ubiquitous proteinaceous narrow-spectrum antibiotics produced by a variety of microorganisms (Farkas-Himsley, 1980). A new bacteriocin, virgicin isolated from a marine bacterium *Virgibacillus* species was evaluated for anti-biofilm activity and found that it is selectively inhibiting the growth of Gram-positive bacteria and biofilm formation by *Enterococcus faecalis* so it could be a promising compound to target oral bacterial species (Gupta et al., 2019).

Several strategies are currently being explored to find a novel way to deal with antibiotic-resistant biofilms. As per the literature review, there are still lots of gaps that need to be filled. One such major gap is to deal with multispecies biofilms *in vivo* where several factors are involved, for instance, intercommunications of species, host factors, etc. Many strategies generate highly promising results on mono-species culture biofilm but are not effective or less significant on multispecies biofilms. The best approach to deal with biofilms is to combine already existing strategies to bring the highly convincing results both *in vitro* and *in vivo* laboratory analysis and also to move a step further towards clinical trials.

1.4. A novel rational strategy to find new anti-biofilm agents and identify their molecular mechanisms of actions

There is a need to formulate novel strategies to find new ways to control or eradicate biofilms and identify their molecular mechanism of actions. A combination of a deep understanding of the biofilms and thoughtful process to find the solutions to target biofilms is the aim of the project.

1.4.1. Molecular targets in *E. coli* biofilm formation and the molecular toolbox for detecting effects on these targets

The major target for most of the compounds in the plant extracts could be the matrix components directly i.e. curli and pEtN-cellulose. Plant extracts can act on the matrix components through two different ways i.e. at the gene level and can disrupt the synthesis of curli and cellulose or can also pose interference in the assembly of the matrix components outside the cell.

The other target of synthesis of matrix components could be the major sigma factor involving in the biofilm formation that is at the top of the hierarchy of the molecular regulatory cascade of the biofilm formation, RpoS. CsgD is the major transcriptional regulator of the curli and cellulose synthesis and also be a strong candidate to be targeted by the anti-biofilm compounds in the plant extracts. Plant extracts can either directly or indirectly act at CsgD by modulating other factors controlling the transcription and/or translation of CsgD such as OmpR, CpxR, cell envelope stress response, heat shock responses, or various sRNA molecules.

The c-di-GMP is another key component that can be targeted by the plant extracts. As it is the essential component that decided the lifestyle of the bacteria under different circumstances so it could be a potential target of the unknown anti-biofilm compounds. In addition to this, the flagellar gene cascade could also have some effects to inhibit the biofilm formation.

1.4.2. Traditional herbal medicine as a source of novel anti-biofilm agents

Plants in general have to develop strategies to prevent the growth of phytopathogens on them. So, to achieve that, plants have to defend these bacteria either by killing harmful bacteria and/or by preventing their colonization by producing some anti-biofilm compounds. Antimicrobial effects of plants and/or their strategies to control their virulence factors are already documented in various studies (Radulović, Blagojević, Stojanović-Radić, & Stojanović, 2013; Silva, Zimmer, Macedo, & Trentin, 2016). On the other hand, enteric bacteria such as *E. coli* occurring

in animals, birds, and humans can release bacteria into the environment (Teplitski & de Moraes, 2018). This might give chance to enteric bacteria to survive on the plant surfaces where they have to face stressful situations such as nutrient deficiency, desiccation, temperature variations, etc. and it has been also reported that curli fibers and pEtN-cellulose are involved in the biofilm formation of enteric bacteria on the plant surfaces (R. Hengge, 2019).

Ayurveda is the system of medicine that evolved in India with a rationale logical foundation (Narayanaswamy, 1981). Indian ayurvedic preparations are useful in general human health and play an important role in modern medicines (Patwardhan, Vaidya, & Chorghade, 2004). Single and polyherbal preparations are cocktails of biologically active compounds and have significant human health benefits since ancient times and can be used to treat various infectious diseases (Tambekar & Dahikar, 2011). So, it could be possible that such medicinal plants may have some anti-biofilm strategies to deal with biofilm-related infections.

1.4.3. Proof-of-principle studies

It has already been documented in recent studies that plant products do have anti-biofilm active compounds such as EGCG and various flavonoids (Pruteanu, Hernández Lobato, Stach, & Hengge, 2020; D. O. Serra et al., 2016). In both the above-mentioned studies, it has been reported that directly targeting the extracellular matrix components seems an attractive strategy as the anti-biofilm compounds do not have to enter the cell wall which could be a barrier for different chemical molecules. Alternatively, such compounds have indirect ways to trigger the bacterial gene expression or intracellular signaling by targeting envelope stress that can be initiated from outside (Pruteanu et al., 2020; D. O. Serra et al., 2016). Notably, EGCG is eliminating the biofilm formation by directly interfering with the assembly of amyloid fibers and also by triggering the cell envelop stress response RpoE and thereby reducing the expression of CsgD whereas flavonoids tend to eliminate biofilm by inhibiting the assembly of curli where interference of pEtN-cellulose is still unknown (D. O. Serra et al., 2016) (Pruteanu et al., 2020)

1.4.4. Preliminary studies leading to this Ph.D. project

One of the most common neurodegenerative disorders characterized by progressive loss of memory is Alzheimer's disease (AD). There are two major neuropathological features for the diagnosis of AD, namely, the extracellular plaque formation comprising amyloid-β protein (Aβ) mainly confined in the hippocampus region of the brain and the cerebral cortex, domains related to memory and other higher cognitive functions and other is neurofibrillary tangles (NFTs)

formation intracellularly (S. K. Singh, Srivastav, Yadav, Srikrishna, & Perry, 2016). Amyloid patterns of protein assembly and folding are highly conserved through evolutions and appears in all the levels of organisms (Friedland, 2015). So, there is a potential chance that plants used in Ayurveda for promoting memory or treatment of various neurodegenerative diseases could have some strategy to deal with amyloid proteins.

2. Goals of the project

Following the rationale mentioned in the previous section 1.4.4, Dr. Ruchira Mukherji (former postdoc in the Hengge group) had already analyzed extracts of 19 Ayurvedic plants that are used to promote memory or prevent or treat Alzheimer's disease or dementia in general. She concluded from her results that several extracts contain very powerful anti-biofilm compounds. This Ph.D. project is in continuation of her preliminary results where 10 highly strong plant extracts have been selected for further studies. The major goals of this project are to reproduce the preliminary results obtained by Dr. Ruchira (unpublished data of Hengge group) with 10 plant extracts with optimized standard procedures to screen the anti-biofilm plant extracts and to elucidate their mode of action. In addition to this, the following were the goals of this project:

- Elucidate the molecular mechanism of action of selected plant extracts and/or pure anti-biofilm compounds
- To test the effect on wider range bacteria covering both Gram-positive and negative pathogenic bacteria both on solid-state biofilms and submerged biofilms
- Further characterize the activities of the most active plant extracts (on the biofilm-related functions, e.g. showing effects in vitro with assembly assay for purified curli subunits CsgA)

3. Materials and Methods

A combination of a microbiological, molecular biological, and enzymatic assay, experiments were done to meet the aims of this project. Ten selected effective plant extracts were prepared under well-standardized conditions and before proceeding towards screening procedures, various physiochemical characterization of extracts was performed to avoid any hindrance in microbiological or molecular, or biochemical analysis later in this project. Initial screening of the anti-biofilm effect with plant extracts was done on petri plates containing LB without salt and supplemented with Congo red. Efforts were also made to identify the broader range of anti-biofilm effects of plant extracts on both Gram-positive and Gram-negative bacteria (including few pathogenic strains also). Lastly, the plant extracts were investigated for their target sites using various molecular and biochemical assays.

3.1. Equipment

Following Table 1 shows the list of equipment used in this study.

Table 1: List of Equipments used

96 well microtiter plates	• Non-treated, flat bottom, non-binding, OPAQUE Geriner • Non-treated, flat bottom, non-binding, transparent Geriner • Human Fibronectin coated 96-Well Microplates R&D SYSTEMS
Autoclave	Technoclav 50 Tecnomara
Blotting Apparatus	EBU-302. C.B.S. Scientific
Cameras	Leica DC 300F/ICc3. Zeiss/Canon 1100D
Centrifuge	• Centrifuge 5417R Eppendorf • Centrifuge 5804R Eppendorf • Minispinplus Eppendorf • Thermoscientific Sorval WX Ultra

Electrophoresis Chambers	Mini/Medi Protean II. BIO-RAD MGV 202-33. C.B.S. Scientific Easy Cast B2 Owl Separation systems
Electroporator	GenePulser Xcell BIO-RAD
Fume Hood	InfraLab
Gel Electrophoresis Power Supply	EPS 600 Pharmacia Biotech
Heating block	Thermomixer Comfort Eppendorf
Hot plate with a magnetic stirrer	VMR
Ice Maker	Ziegra EISmaschinen
Incubators	Memmert
Laminar Air Flow	Safe 2020
Nanodrop	NanoDrop 2000 PeqLab
pH meter	SI analytics
Plate Reader	BioTek, Synergy/H1 microplate reader
Rotatory evaporator	BÜCHI Rotavapor R-114
Shakers	GFL
Spectrophotometer	Biochrom
SpeedVac	Thermoscientific
Stereomicroscopy	Leica S8 AP0/Stemi 2000-C. Zeiss
TEM Grids, FF 200-Ni-TH Formvar film Square mesh	Electron Microscopy Sciences
Vacuum pump	IntergaVacusip
Vortex mixer	Voatex2 genie
Water bath	HT Infors AG

3.2. Chemicals

The following are the recipes used for preparing different media and buffers used in microbiological, molecular biology techniques, and biochemical assays. All the chemicals were handled in the laboratory and disposed of as per the guidelines stated in the MSDS (Material Safety Data Sheet) provided by the manufacturer or as per the standard guidelines formulated by the Institute.

A. Media used in Microbiology work

- **Luria-Bertani-Medium (LB)**

Bactro- Trypton	**10 g**
Bactro- Yeast extract	**5 g**
NaCl	**5 g**
Water	**1 L**

The medium was sterilized in an autoclave

- **Luria-Bertani-Agar-Medium (LB-agar)**

Bactro- Trypton	**10 g**
Bactro- Yeast extract	**5 g**
NaCl	**5 g**
Span Agar	**18 g**
Water	**1 L**

The medium was sterilized in an autoclave

- **Luria-Bertani-Agar-Medium (LB-Agar) without salt**

Bactro- Trypton	**10 g**

Bactro- Yeast extract	**5 g**
Span-Agar	**18 g**
Water	**1 L**

The medium was sterilized in an autoclave

- **Congo Red solution** (in Ethanol 70%)

Congo Red	**2 mg/mL**
Coomassie-Brilliant-Blue G	**1 mg/mL**

The solution was filter sterilized and stored in the fridge at 4 deg. C in a dark bottle

- **Luria-Bertani-Agar-Medium (LB-Agar) without salt supplemented with Congo Red solution**

Bactro- Trypton	**10 g**
Bactro- Yeast extract	**5 g**
Span-Agar	**18 g**
*Congo Red solution	**20 mL**
Water	**1 L**

*Congo Red was added after autoclaving the medium. Dispense 3 mL of medium in 35 mm tissue culture dishes. Plates were kept on bench for 2 days before inoculating them.

- **TSB (Tryptic Soya Broth) supplemented with Agar, 100 mM $MgCl_2$ and Congo Red solution**

Tryptic Soya Broth **(BD Bacto™)**	**As per stated on the label**
*Anhydrous $MgCl_2$	**100 mM**
Span-Agar	**18 g**

*Congo Red solution	**20 mL**
Water	**1 L**

*Congo Red and $MgCl_2$ were added after autoclaving the medium. Both the solutions were sterilized separately using syringe filtration techniques. $MgCl_2$ can be stored at room temperature. Dispense 3 mL of medium in 35 mm tissue culture dishes. Plates were kept at ambient temperature for 2 days for drying before inoculating them.

- **TSB (Tryptic Soya Broth) supplemented with Agar, 100 mM $MgCl_2$ and 0.25% Dextrose solution and 2.5 % NaCl**

Tryptic Soya Broth **(BD Bacto™)**	**As per stated on the label**
*Anhydrous $MgCl_2$	**100 mM**
Span-Agar	**18 g**
*Dextrose	**2.5 g**
NaCl	**25 g**
Water	**1 L**

*Dextrose and $MgCl_2$ were added after autoclaving the medium. Both the solutions were sterilized separately using syringe filtration techniques. $MgCl_2$ can be stored at room temperature and dextrose can be stored in the fridge (avoid too long storage). Dispense 3 mL of medium in 35 mm tissue culture dishes. Plates were kept at ambient temperature for 2 days for drying before inoculating them.

- **X-Gal-Agar:**

  To autoclaved LB-agar medium, 40 mg/L 5-bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside (X-Gal dissolved in N,N-dimethylformamide) was added. Syringe filtered separately before adding to sterilized LB-agar.

B. Buffers and solutions used in Plasmid Gel Electrophoresis

- **Gel application buffer (6X)**

Bromophenol Blue	**0.25%**

Xylene cyanol	**0.25%**
Glycerin	**30% dissolved in 1X TAE buffer**

- **TAE buffer (50X)**

Tris	**242 g**
Acetone	**57.1 mL**
0.5 M EDTA pH 8	**100 mL**

C. Buffer used in Beta-Galactosidase assay

- **Z-Buffer (5X)**

Na_2HPO_4. $7H_2O$	**80.5 g**
NaH_2PO_4. H_2O	**27.5 g**
KCl	**3.75 g**
$MgSO_4$. 7 H_2O	**1.23 g**
Double Distilled water	**1 L**

D. Buffers and solutions used in SDS-Polyacrylamide-Electrophoresis

- **Lower and Upper Buffer**

Lower Buffer		**Upper Buffer**	
Tris	**181.7 g**	Tris	**60.6 g**
SDS 20%	**20 mL**	SDS 20%	**20 mL**
Adjust pH with HCl	**8.8**	Adjust pH with HCl	**6.8**
Water	**1 L**	Water	**1 L**

- **Separating and Stacking Gel**

12 % Separating Gel		**4% Stacking Gel**	
Lower buffer	**2.5 mL**	Upper buffer	**1.25 mL**
Acrylamide stock	**4.0 mL**	Acrylamide stock	**0.65 mL**
MilliQ water	**3.45 mL**	MilliQ water	**3.07 mL**
10% APS	**50 microliters**	10% APS	**25 microliters**
TEMED	**5 or 10 microliters**	TEMED	**5 or 10 microliters**

- **Sample Buffer (4X) and SDS-PAGE-Electrophoresis Buffer**

4X Sample Buffer		**SDS-PAGE-Electrophoresis Buffer**	
Tris-HCl pH 6.8 (AppliChem)	**240 mM**	Tris-HCl	**25 mM**
SDS solution (AppliChem)	**8%**	Glycerin	**190 mM**
Glycerin	**40%**	SDS	**0.1%**
DTT	**400 mM**		
Bromophenol Blue	**0.02%**		

- **Running Buffer (10X)**

Running Buffer	
Tris	**30 g**
Glycin	**144 g**
SDS 20%	**50 mL**
Water	**1 L**

- **Gel Staining Solution**

Coomassie-Gel staining solution (in water)	
Isopropanol	**25%**
Acetic Acid	**10%**
CoomassieBrilliant Blue G	**0.05%**

- **De-staining solution: 10% Acetic acid**

E. Buffers for Immunoblot analysis

- **TBST Buffer**

1M Tris pH 7.5	**20 mL**	Final conc.: 20 mM
5M NaCl	**30 mL**	Final conc.: 150 mM
Tween 20	**500 µL**	Final conc.: 0.05 %
Water up to	**1000 mL**	

- **TBST Milk Buffer: 5 % milk powder in TBST**
- **Transblot Buffer**

Tris pH 7.5	**25 mM**
Glycerin	**192 mM**
Ethanol	**20%**

- **AP Buffer**

1 M Tris pH 9.5	**50 mL**	Final conc.: 100mM
5 M NaCl	**10 mL**	Final conc.: 100mM
1 M $MgCl_2$	**2.5 mL**	Final conc.: 5 mM

Water upto	**500 mL**	

F. Buffers and solutions used in CsgA polymerization assay

- **Potassium phosphate buffer (KPi Buffer):**

Potassium phosphate buffer 50 mM, pH 7.2 (in water)	
KH_2PO_4	28.9 mM
K_2HPO_4	21.1 mM

Store at 4 deg. C

- **Elution Buffer 1:**

Elution Buffer 1 (in Potassium phosphate buffer)	
Imidazole	100 mM

Store at 4 deg. C

- **Elution Buffer 2:**

Elution Buffer 2 (in Potassium phosphate buffer)	
Imidazole	500 mM

Store at 4 deg. C

3.3. Bacterial Strains

Table no. 2 shows a series of *E. coli* bacterial strains that were used to screen the anti-biofilm effect of plant extracts, to study their molecular mechanisms, and to do various other molecular or biochemical assays. This table contains complete information about the strains such as their background strain, phenotype, type of mutations, and references. *E. coli* K-12 strain W3110 (Jensen, 1993; Serra D.O., Richter A.M., and Hengge R, 2013) produces concentric rings on LB without salt medium supplemented with Congo red. In addition to concentric rings, it also produces the intense red color in the presence of amyloid Congo red dye. This particular strain is producing one of the major components of biofilm of *E. coli* i.e. curli fibrils. Likewise, AR3110 (Serra D.O., Richter A.M., and Hengge R, 2013) is a derivative of W3110 producing both curli fibrils and pEtN-cellulose and shows extremely large, flat with radial ridges red color

(lighter than W3110) colony morphology. A derivative of AR3110 is AR282 with *Csg*B deletion mutation which is producing only pEtN-cellulose which can also bind with the Congo red and produces intertwined wrinkles and stains pink on Congo red plates. The fourth strain used in this series was AR198, a derivative of AR282 in which *bcs*A deletion has been done to produce a phenotype producing expressing neither curli nor pEtN-cellulose and thus it is not staining with Congo Red and producing dirty white colonies. To study the molecular mechanism a range of bacterial strains were used which are shown in Table 2 with detailed information.

Table 2: List of Bacterial strains used

Strain	**Background strain**	**Relevant Genotype**	**Relevant Phenotype**	**References**
W3110	K-12	thyA36, deoC2, IN(rrnD-rrnE)I	Only curli	Jensen, 1993; Serra D.O., Richter A.M. and Hengge R, 2013
AR3110	W3110	Point mutant in front of bcsQ ORF (in 5'-UTR)	pEtN-Cellulose and curli	Serra D.O., Richter A.M. and Hengge R, 2013
AR282	AR3110	*csgB*::scar	Only pEtN-cellulose	Richter A.M. et al, Unpublished
AR198	AR3110	*bcsA*::scar and *csgB*::cm	No curli; No pEtN-cellulose	Richter A.M. et al, Unpublished
GB1100	W3110Δlac(I-A)	λRS45(*csgB*::*lacZ*)	*csgB* gene is fused with reporter gene lacZ	Klauck G et al, Unpublished

NS252	W3110Δlac(I-A)	IRS74 *yaiC*::*lacZ*(hybr.)	*dgcC* gene is fused with reporter gene *lacZ*	Sommerfeldt N, et al, Unpublished
AR32	W3110Δlac(I-A)	*rpoS*::scar	RpoS negative	Richter A.M. et al, Unpublished
GBK5	W3110Δlac(I-A)	Δ*csgD2*::scar	CsgD negative	Klauck G et al, Unpublished
GB1027	W3110Δlac(I-A)	*synP8*::*lacZ*	*synP8* gene is fused with reporter gene *lacZ*	Klauck G et al, Unpublished
AP586	W3110Δlac(I-A)	λRS45 degP(-129,+19; K2->S,K3->A)::lacZ hybrid	*degP* gene is fused with reporter gene *lacZ*	Possling A et al, Unpublished
RON1	W3110Δlac(I-A)	*degP*::lacZ; *cpxR*::kan	*degP* gene is fused with reporter gene *lacZ* and *cpxR* mutant	Offer R et al, Unpublished
AP587	W3110Δlac(I-A)	*bdm*::lacZ	*bdm* gene is fused with reporter gene *lacZ*	Possling A et al, Unpublished
RON3	W3110Δlac(I-A)	*bdm*::lacZ; *rcsC*::cat	*bdm* gene is fused with reporter gene *lacZ*	Offer R et al, Unpublished

			and *rcsC* mutant	
RON2	W3110Δlac(I-A)	*bdm*::lacZ; *rcsB*::kan	*bdm* gene is fused with reporter gene *lacZ* and *rcsB* mutant	Offer R et al, Unpublished
AP562	W3110Δlac(I-A)	*dnaK*::lacZ	*dnaK* gene is fused with reporter gene *lacZ*	Possling A et al, Unpublished
KL25	W3110Δlac(I-A)	*fliA*::lacZ	*fliA* gene is fused with reporter gene *lacZ*	Lenz K et al, Unpublished
NS256	W3110Δlac(I-A)	IRS45 *yhjH*::lacZ (hybr.)	*yhjH* gene is fused with reporter gene *lacZ*	Sommerfeldt N, et al, Unpublished
VP385	W3110Δlac(I-A)	*csgB*::lacZ , *ydaM*::scar , *yciR*::kan	*csgB* gene is fused with reporter gene *lacZ* and *ydaM* and *yciR* mutant	Pfiffer V et al, Unpublished
EAEC-55989 (Enteroaggregative *Escherichia coli)*	-	-	-	(Touchon et al., 2009)

UPEC-CFT073 (Uropathogenic *Escherichia coli)*	-	-	-	(Welch et al., 2002)
Pseudomonas aeruginosa UCBPP-PA14	-	-	-	(D. G. Lee et al., 2006)
Bacillus subtilis NCIB 3610	-	-	-	(Nye, Schroeder, Kearns, & Simmons, 2017)
Staphylococcus aureus	-	-	-	(Baba, Bae, Schneewind, Takeuchi, & Hiramatsu, 2008)
Escherichia coli (LSR12/pMC1)	*Escherichia coli* C600	LSR12: *csgDEFG csgBA* deletion mutant and Plasmid pMC1 was made by cloning CsgG into the *Nco*l and *Bam*HI sites of pTrc99A	Expressing protein CsgG, involved in extracellular localization and assembly of curli subunits	(Zhou, Smith, Hufnagel, & Chapman, 2013)
Escherichia coli (pMC3)	*Escherichia coli* C600	Plasmid pMC3 cloned with full length C-terminal His_6-tagged CsgA into *Nde*I and	Expressing His_6-tagged CsgA subunits	(Zhou et al., 2013)

		*Eco*RI sites of pHL3		

3.4. Microbiological working and methods

This section covers all the working conditions used in microbiological work such as sterilization techniques and conditions, growth conditions, storage of bacterial strains, concentrations of antibiotics used, the basic procedure to determine cell growth using colony-forming units, and optical cell density of liquid culture using a spectrophotometer.

3.4.1. Sterilization

Both liquid media and solid media were sterilized by autoclaving for 20 min at 121^0C at 1 bar pressure. Heat-labile solutions were filtered sterilized using pEtN-cellulose filters from Roth (pore size 0.22 microns).

3.4.2. Growth conditions

Bacteria were grown both in liquid cultures and solid media either at 28^0C or 37^0C depending upon the type of bacterial strain and as per the requirement of the experiments. Aerobic conditions in the liquid culture were ensured by incubating culture flasks or test tubes at 200 rpm.

3.4.3. Storage of bacterial strains

Bacterial strains were streaked on LB-agar plates and incubated at 37^0C overnight. A single colony was inoculated in 5 mL of sterilized LB broth and incubated at 37^0C under shaking conditions overnight. With 7% Dimethyl sulfoxide (DMSO) in 1 mL activated overnight culture were mixed in screw-capped cryovials and stored at -80^0C for long term preservation. For short term preservation, a freshly streaked culture can be stored in the fridge at 4^0C for 10-15 days.

3.4.4. Activating bacterial culture

Bacterial strains were streaked onto LB plates and incubated at 37^0C overnight. A single colony was picked and inoculated in fresh sterile 5 mL medium (LB or LB without salt as per the requirement of the experiment) in a test tube and incubated at 37^0C under shaking conditions

overnight. The start culture of OD=0.05 at 578 nm was prepared from activated culture to do further microbiological experimentations.

3.4.5. Antibiotics

In this specific project, only two antibiotics had been used i.e. Ampicillin and Chloramphenicol.

The stocks of both the antibiotics were stored at -20^{0}C. Before using, stocks were thawed on ice and further used for preparing working solutions. Care while handing these antibiotics were followed as per the MSDS (Material Safety Data Sheets) provided by the manufacturer. Following Table no. 3 shows the concentrations of the stocks and working solutions.

Table 3: List of antibiotics used

Antibiotics	Stock concentrations	Working concentrations
Ampicillin	100 mg/mL	100 µg/mL
Chloramphenicol	20 mg/mL in 70% Ethanol	20 µg/mL

3.4.6. Determination of cell density of the liquid bacterial culture

The determination of cell density in the liquid cell was done spectrophotometrically at 578 nm. The sterile medium was used as a reference. Since the cell density and optical density are not linear after 0.3 value so, the samples were diluted with a reference medium as per needed, and actual cell density is counted by multiplication with dilution factor.

An additional control, plant extracts/purified compound without any bacterial strain was also used when working with plant extracts or purified compounds and OD values of the culture in the presence of extract were adjusted accordingly.

3.4.7. Colony-forming units (CFU)/mL

For colony-forming units per milliliter, 20µL of the sample (bacterial culture) was added to 180µL of reference medium in a row in 96-well plate. Sterile reference medium was used as a negative control. The first well was mixed by pipetting 20µL up and down, 20 times. Then 20µL was transferred from the first well to second without plunging the tip all the way and then mixed with a fresh sterile tip. 20µL from the second well was transferred to the third well as above and so on to the eighth well. The result was a serial dilution of the sample from a dilution

10^{-1} (first well) to 10^{-8} (last well). From each dilution, three spots of 20µL were plated on LB medium and done in duplicates. Once the plates get dried, incubated at 37^0C overnight. The dilution having 30-100 colonies was selected and counted from each spot and the mean was used for calculating the cfu/mL using the following formula:

CFU (colony forming unit)/mL = (Average number of colonies) x 1000 x (Dilution factor)/ Volume of culture spotted/plated (µL)

3.5. Preparation of crude plant extracts

Table 4 listed the plant material used in this study with complete information including the botanical name of plants, common name (Sanskrit/Hindi name), plant part used, commercial supplier, and the optimized volumes of plant extracts used in this study.

Table 4 List of plants used and related information

Scientific name	Sanskrit/ Hindi name	Plant part used	Commercial supplier	Amount of the crude extract per 3 mL of medium/per plate
Tinospora cordifolia	Guduchi/ Amritavalli	Whole plant	Narayani Naturals, Mohan Cooperative Industrial Estate, Mathura Road, New Delhi-110044	60 µL
Cinnamomum zeylanicum	Darusitha/ Dalchini	Tree bark	Store bought	20 µL
Garcinia mangostana	Vrikshamla/ Kokam	Fruit	Mittal Ayurved Sansthan, Mansarovar Industrial Estate ext., Merrut, India. www.3gorganic.com	10 µL

Bacopa monnieri	Brahmi	Whole plant	Biofeel, Empress-Augusta-Str. 69, Berlin, Germany, 12103	60 µL
Commiphora whighitti	Guggulu	Oleogum resin	Royal Herbal Land Pvt. Ltd. Lonavala co. op industrial Estate Ltd Lonavala, Pune 410401. www.herbalhills.in	60 µL
Terminalia chebula	Haritaki	Fruit	Narayani Naturals, Mohan Cooperative Industrial Estate, Mathura Road, New Delhi-110044	10 µL
Glycyrrhiza glabra	Yashtimadhu /mulethi	Roots	Narayani Naturals, Mohan Cooperative Industrial Estate, Mathura Road, New Delhi-110044	60 µL
Fagonia arabica	Dhamasa/ Durlabha	Above ground parts	Royal Herbal Land Pvt. Ltd. Lonavala co. op industrial Estate Ltd Lonavala, Pune 410401. www.herbalhills.in	60 µL
Acorus calamus	Vacha	Rhizome	Sierra India Oganics, Indore-452001, India	60 µL
Aegle marmelos	Shivaphala/ Bael	Fruit, Leaves	Royal Herbal Land Pvt. Ltd. Lonavala co. op industrial Estate Ltd Lonavala, Pune 410401. www.herbalhills.in	60 µL

2 gm of dried powdered plant material or crushed seeds, barks, etc. was weighed in a sterile 50 mL falcon tubes. To this, 50 mL of 1:1 mixture of pure ethanol: double distilled water was added. Tubes were mixed well by vortexing and then incubated in a water bath pre-set at 70 deg. C for 30 mins with shaking at 100 rpm. Intermittent mixing by vortexing was done to enhance the extraction of bioactive constituents. Following the heat treatment, the tubes were kept on the bench at room temperature for a cool down and left overnight at 4^0 C to finish the extraction process. The following day, tubes were centrifuged at 8000 rpm for 25 mins to obtain clear supernatant which was further used to make a concentrated crude extract.

To prepare the concentrated crude extract, samples were dried on a rotatory evaporator (BÜCHI Rotavapor R-114). Preset the water bath of the rotatory evaporator at 45°C and condenser at 7°C to speed up the evaporation. The pressure was regulated as per the suitability of the sample as some samples form foam during the process of concentration. The volume of the sample was reduced from 50 mL to nearly 10 mL or less if possible and further concentrated or dried preferentially on speedvac using 2 mL ependorffs. To this dried material, 2 mL of 1:1 mixture of pure ethanol: double distilled water was added and mixed properly by vortexing. The prepared and concentrated crude extract was then stored at 4^0C until further use. (NOTE: some of the plant extracts get contaminated with time or may lose their activity, so, it is always advisable to replace the old stocks with fresh extracts).

3.5.1. Physico-chemical characteristics and sterility test of plant extracts

Physical and chemical characteristics of plant extract such as a change in pH (using pH strips), color change, and formation of precipitates were also observed before and after concentrating the extract. To test the sterility of extract, 50 µL of the extract was mixed with 50 µL of sterilized water under aseptic conditions and was spread onto LB agar. 100 µL of sterilized water was used as control. Plates were incubated at both 28^0C and 37^0C for 3-4 days. No growth ensured the sterility of plant extracts.

3.5.2. Effect of Plant Extracts on pH of working medium

To ensure if the pH of the extract was not bringing any change in the pH of the working LB growth medium, optimized working concentrations of plant extracts were added to the growth medium and pH change was checked using pH strips. (NOTE: pH meter cannot be used for this purpose because it is not possible to prepare such solutions in bulk as the volume of plant extracts were very low after concentrating).

3.5.3. Growth curves with and without plant extracts in liquid culture

Figure 2 shows a schematic diagram of sampling for growth curves where PE stands for Plant extracts and LBon stands for LB medium without salt. Plant extracts (optimized concentrations; procedure explained in the following sections 3.5.5) were dried completely before they were used (to remove ethanol and water) by leaving appropriate volume at 37^0C overnight in 50 mL of the conical flask. To this, 5 mL of LB without salt was added (two such sets were prepared separately). One set was inoculated with activated bacterial culture (as stated in section 3.4.4) prepared in LB without salt and the other set is left uninoculated as control.

LBon (LB without salt) without plant extract was also prepared in two different sets. To one set, inoculation was done with respective bacterial strain and the other was left uninoculated as control. Both these sets were controls of the experiments. All the flasks along with their respective controls were incubated at 28^0C for 24 hr at 200 rpm in a water bath. Sampling for growth curves was done at 4^{th} hr, 6^{th} hr, 8^{th} hr, 10^{th} hr, 12^{th} hr, and 24^{th} hr. Cell growth was analyzed using a spectrophotometer as explained in section 3.4.6.

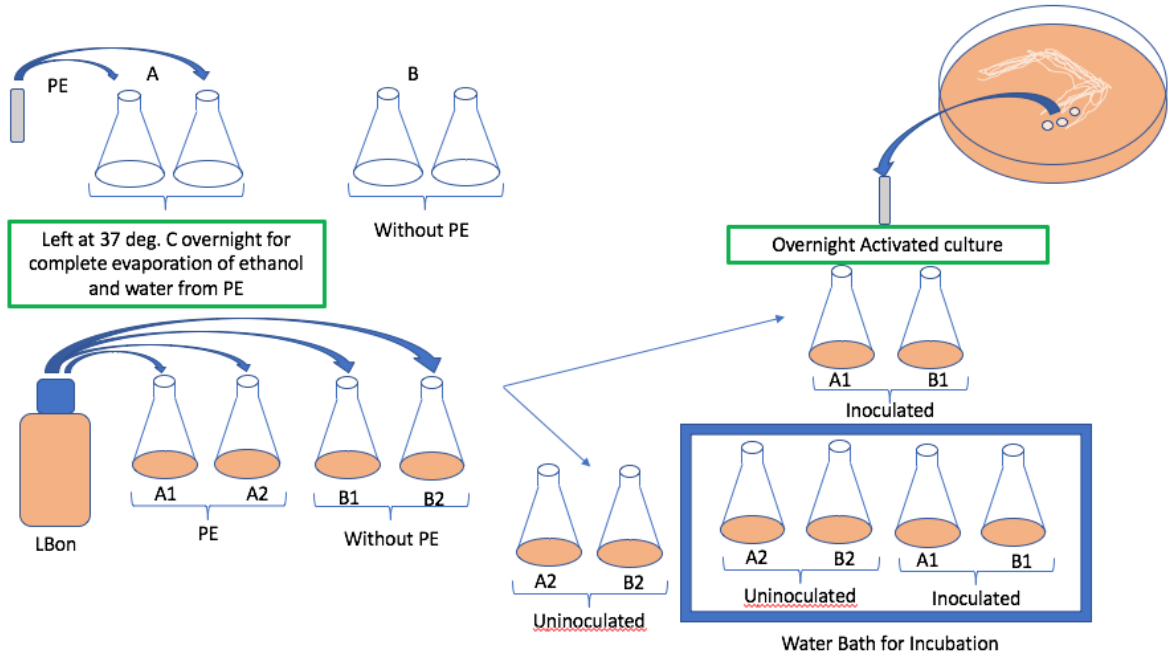


Figure 2: Schematic diagram of sampling for growth curves. PE stands for Plant Extract and LBon stands for LB without salt

3.5.4. Preparation of LB-Agar without salt (LBon) agar plates supplemented with Congo red for macrocolony morphology

Figure 3 illustrates the basic schematic diagram for preparing the LBon plates supplemented with Congo red (D. O. Serra & Hengge, 2017). To sterilized LBon medium, Congo red added (concentrations stated in section 3.2) and 3 mL of medium was dispensed in 35 mm tissue culture plates. Plates were kept on a bench for 2 days for drying before inoculating them. The overnight activated culture was prepared in LB medium as mentioned in section 3.4.4. Spotting of 5 µL of activated culture was done in the middle of the plates. Plates were left on the bench till the inoculum gets adsorbed onto the medium. Later, plates were incubated at 28^0C for 3-5 days. Changes in colony morphology were observed under stereomicroscope and images were taken using ICc3. Zeiss camera.

For testing plant extracts for their anti-biofilm effects, LBon plates with Congo red were supplemented with plant extracts (concentrations mentioned in section 3.5.5 and Table 4). A set of four strains W3110 (only curli), AR3110 (both curli and pEtN-cellulose), AR282 (only pEtN-cellulose), and AR198 (no curli; no pEtN-cellulose) were used because of their distinctive colony morphologies (description mentioned in section 3.3 and Table 2) on LBon medium to screen anti-biofilm effects of plant extracts. Plates without plant extract were used as control.

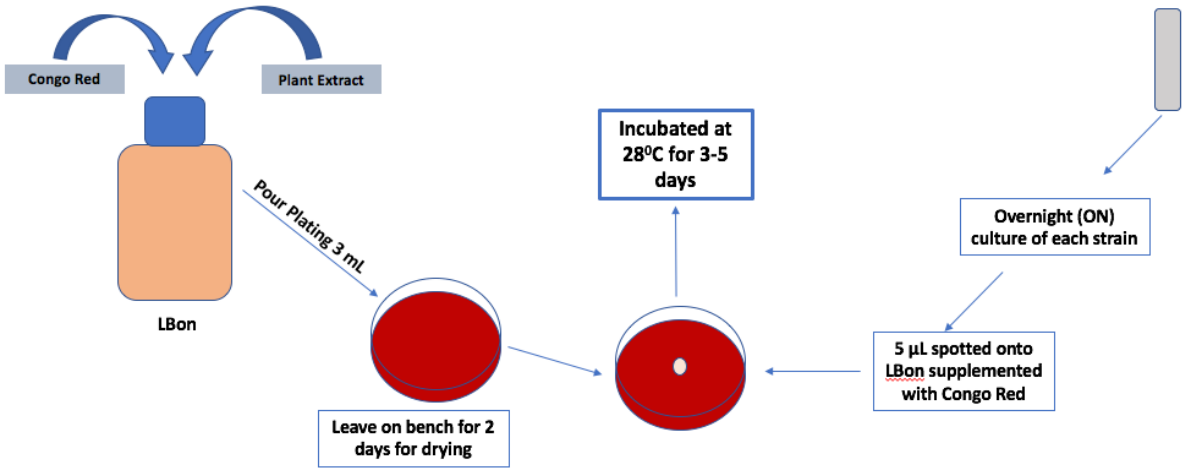


Figure 3: Basic schematic diagram for preparing LBon plates supplemented with Congo Red along with Plant Extracts. NOTE: plates should be closed while left for drying for 2 days on bench.

3.5.5. Optimization of concentration of plant extracts for the screening of only anti-biofilm effects without any anti-bacterial effect

To optimize the concentration of plant extracts, different test concentrations such as 5 µL, 10 µL, 20 µL, 40 µL, 60 µL was added to each 3 mL of Congo-Red LBon Agar plate as explained in the previous section 3.5.4. Each test concentration was inoculated with four sets of bacterial strains (one strain for each plate) mentioned in previous sections and incubated for 3-5 days. Images were taken on a stereomicroscope using ICc3. Zeiss camera. Concentrations at which there was an only anti-biofilm effect without any bacterial growth inhibition were selected for further experimentation.

3.5.6. To study the wide range anti-biofilm activity of plant extracts on different bacterial species

To study the wider range of anti-biofilm effects of plant extracts, both Gram-positive and Gram-negative bacterial species were analyzed (Table 2 and section 3.3). EAEC-55989 (Enteroaggregative *Escherichia coli*) (Touchon et al., 2009), UPEC-CFT073 (Uropathogenic *Escherichia coli*) (Welch et al., 2002), *Pseudomonas aeruginosa* UCBPP-PA14 (D. G. Lee et al., 2006), *Bacillus subtilis* NCIB 3610 (Nye et al., 2017) and *Staphylococcus aureus* (Baba et al., 2008) were used to screen the anti-biofilm effect of plant extracts on these bacteria. Screening was done the same way as mentioned in the section 3.5.4 using optimized concentrations of plant extracts. This particular experiment was performed at both 28^0C and 37^0C. Images were taken under a stereomicroscope.

3.5.7. Optimization of medium and temperature for the study of the anti-biofilm effect of plant extracts in case of *Staphylococcus aureus*

For optimization of medium for *Staphylococcus aureus*, different types of medium compositions were tested. Slightly modified TSB (Tryptic Soya Broth) supplemented with Agar, 100 mM $MgCl_2$ (García-Betancur et al., 2017; Wermser & Lopez, 2018) both with and without Congo Red solution and TSB (Tryptic Soya Broth) supplemented with Agar, 100 mM $MgCl_2$ and 0.25% Dextrose solution and 2.5 % NaCl without Congo red were tested (Cassat, Lee, & Smeltzer, 2007) (recipes mentioned in section 3.2).

To observe wrinkled colonies, both 28^0C and 37^0C incubation temperatures were tested. The basic procedure for macrocolonies was the same as that mentioned in section 3.5.4 except medium composition and temperature conditions were different.

3.6. Effects of plant extracts on submerged biofilms: the crystal violet assay

A slightly modified crystal violet biofilm assay (G. A. O'Toole, 2011) was performed to determine the effect of anti-biofilm constituents of plant extracts on submerged biofilms. The activated overnight culture described in section 3.4.4 was diluted to 1:100 in fresh LB medium. 200 µL of the diluted sample treated with plant extract was added in 96 well plate (Roth) in triplicates. In the case of *S. aureus,* a human fibronectin-coated 96 well microtiter plate had been used (R&D systems a biotechne brand). Untreated LB (without plant extracts) and uninoculated LB (with plant extracts) were used as control. The plate was sealed with "Breathe Easy Membrane" (Merk) and incubated at 28 deg. C. (at 37 deg. C with pathogenic strains) for 24 hrs without shaking. To ensure bacterial growth on 96 well plate in the presence and absence of plant extract, optical density was observed at OD-578 nm on plate reader (BioTek, Synergy/H1 microplate reader). After incubation, the spent medium was removed from each well. Wells were washed 3 times with 300-400 µL of double-distilled water (ddH_2O) and the plate was dried on a bench at room temperature for 30-45 mins. To dried plate, 0.1% crystal violet (in ddH_2O) was added to each well and stained for 10-15 mins at room temperature. Crystal violet was removed after staining and the plate was washed 3 times and dried the same way as described above. The bound crystal violet was then solubilized by adding 300 µL 80:20 Ethanol:Acetone to each well for 30-45 min. Crystal violet was then quantified by measuring the absorption of the solution at 595 nm with the plate reader (BioTek, Synergy/H1 microplate reader).

3.7. Assays used to study anti-biofilm effects of the plant extracts at the molecular level in *E. coli*

Figure 4 shows the schematic plan to study the molecular mechanism of unknown compounds of plant extracts. The basic description of all the above-mentioned mutations containing strains is given in Table 2. Expression level and/or activity of the targeted gene was analyzed by two strategies: (1) by using a reporter strain containing single copy lacZ fusion (the expression of the target gene whose upstream regulation sequences were previously fused to lacZ reading frame) and performing beta-galactosidase assay (F. Miller, 1972) and (2) by doing Western Blotting. Following is the stepwise procedure.

3.7.1. Sampling

Sampling for beta-galactosidase assay and western blotting was done the same way as mentioned in section 3.5.3 with an exception in a variation of time of sampling. Sampling for

beta-galactosidase assays (to check the expression activity of the target gene product) and western blotting (to check the expression level) in case of RpoS was done at the 24^{th} hour. To check the cellular expression level of CsgD using western blotting, samples were collected at 8^{th} hr, 10^{th} hr, 12^{th} hr, and 24^{th} hr (this was designed in such way because of the shorter life span of protein CsgD).

All the samples were collected in 1.5 mL eppies (amount of sample collection depends on the expression level of the target gene on X-Gal plates; it is just a guess estimation of proceeding further in the next steps. If you suspect low expression level, then 1 mL is enough otherwise 500µL is fine). Samples were centrifuged at 14,000 rpm for 10 mins and the supernatant was discarded. Pellet was resuspended in 1X Z-buffer. Centrifuged again and the final obtained pellets were stored at -20^0C. Washing of pellet with 1X Z-buffer was necessary to avoid interference of medium components or plant material components in the beta-galactosidase assays.

3.7.2. Determination of lacZ gene fusion reporter expression using beta-galactosidase assay (F. Miller, 1972)

Preserved cell pellets were resuspended in 1x Z-buffer and diluted if necessary (depending on the expression level of a gene) was further used in this assay. An appropriate amount of cell suspension was used and the final volume was made to 1 ml with 1x Z-buffer. A test approach without cell suspension and plant extracts without bacterial inoculation was considered as reference. To each tube, 2 drops of chloroforms and one drop of 0.1% of SDS was added and vortexed for 5 sec and further incubated for 5-10 min at room temperature. 200 µL of freshly prepared ONPG (4 mg/mL Milli-Q water) was added to each tube considering this time sample as time=0 (t_o). This step was repeated every 10 sec for every new sample. The reaction was stopped when it started to get yellow by adding 500 µL 1M Na_2CO_3. This time was considered as t_f and Δt (t_f- t_o) was calculated. The reaction mixture was added to 2 mL Eppendorf and centrifuged for 5 min at full speed (14000 rpm). 300 µL of the supernatant of each sample was placed into a microtiter plate and absorbance was measured at 415 nm. Using the following formula specific activity of beta-galactosidase activity was calculated.

$$\mathbf{A = (3.38 \times E_{415})/(\Delta t \times mL\ of\ cell\ suspension\ in\ assay \times OD_{578})}$$

3.38= a constant associated with the absorption of the ONPG

E_{415}= absorbance at 415 nm of the final reaction

Δt= time difference between addition of ONPG and Na_2CO_3

OD_{578}= OD of bacterial cell suspension in 1X Z-buffer

Single value data are the average of at least three biological replicates.

3.7.3. Determination of protein level using Western Blotting

RpoS and CsgD expression levels were determined by SDS-PAGE, a technique of separating proteins according to their molecular weight using acrylamide gel (Laemmli, 1970), followed with blotting. The recipes of all the chemicals and buffers used in this technique are mentioned in section 3.2. SDS-PAGE was further subjected to Immunoblot-analysis. Cell pellets collected were resuspended in 1X loading buffer to get approximately 1 µg/µL. Samples were heated at 100^0C for 10 mins. This could be used directly for SDS-PAGE or be stored at -20^0C.

3.7.3.1. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Gel preparation recipe and buffers used are explained in Section 3.2. Ten (10) µg of protein from each sample was loaded into the wells. 3-5 µL of Pre-stained Chemiluminescent Protein Ladder (LI-COR) was used as a reference. Proteins were finally separated into the SDS-PAGE Electrophoresis buffer at 25mA for 1 hour. RpoS negative and CsgD negative mutants were used as negative controls of the experiment.

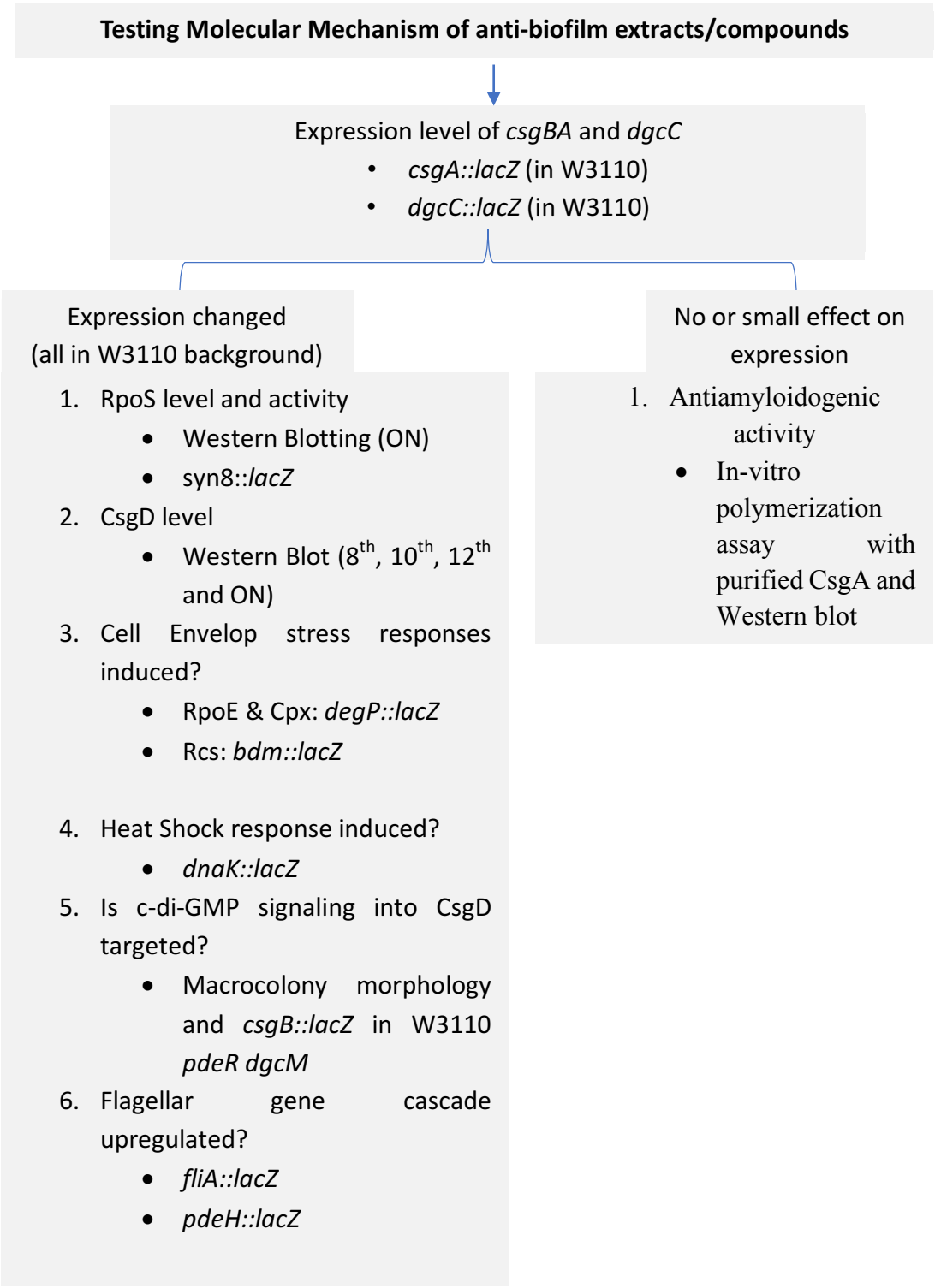


Figure 4: Schematic plan to study the molecular level target(s) of unknown bioactive compounds in plant extracts

3.7.3.2. Sensitive coomassie staining of SDS Gels

The coloring of SDS polyacrylamide gels was done in the coomassie solution (recipe mentioned in section 3.2) for 30 mins at room temperature. To make protein bands visible, the gel was subsequently discolored with a 10% acetic solution. Qualification of proteins was done with the (LI-COR) Chemiluminescent Protein Ladder.

3.7.3.3. Immunoblot analysis

Non-specific staining of all proteins by means coomassie was not intended, samples could also be specifically detected by specific antibodies. To ensure this, proteins were transferred to a PVDF membrane (ROTH) after electrophoretic separation. After equilibration of these in ethanol, water, and transblot buffer, the membrane was fixed together with the protein gel and Whatman papers in the blotting apparatus and blotted for 75 mins at 100 V in cold transblott buffer. The blocking of non-specific protein binding sites was done by incubation in TBST milk buffer overnight at 4^0C. The incubation with the primary antibody (1:10,000 in TBST milk buffer) was done for 3 hours. After 3 washing steps in TBST buffer (every 10 mins), membranes were treated with anti-rabbit horseradish peroxidase-linked species-specific whole antibody (from donkey) (Amerchan ECL^{TM}). Incubated for 1-2 hr and wash with TBST for 15 mins at room temperature and removed. Fresh TBST was added and incubated for 5 mins and removed (done twice). Clarity^{TM} Western ECL substrate (BIO-RAD) was prepared in 1:1 ratio and the membrane was subjected to it for 2-3 mins and the solution was removed. Images were taken without any delay using CCD camera imaging.

3.7.4. *In vitro* amyloidogenesis of CsgA

This biochemical analysis was performed to test the anti-amyloidogenic activity of the unknown bioactive compounds of the plant extracts. Following is the stepwise description of the procedure.

3.7.4.1. Plasmid Isolation

Plasmid pMC3 was isolated using Qiagen rapid isolation plasmid prep kit. The procedure was followed as provided by the manufacturer. The purified plasmid was cross-checked by gel electrophoresis and quantified using the nanodrop technique. The sample was stored at -20^0C. This purified plasmid was further used to transform LSR12/pMC1 strain.

3.7.4.2. Preparation of Electro-competent cells

Overnight culture of LSR12/pMC1 was prepared in 5 mL LB supplemented with ampicillin. 250 mL of LB medium in two separate 1 L conical flasks were inculcated with 2.5 mL of overnight activated culture. Incubated at 37^0C at 200 rpm till OD-578 reaches to 0.5. The culture was centrifuged at 6000 rpm at 4^0C for 15 mins to collect the pellet. From this step, onwards, everything done, used, or added was ice cold. Pellet was resuspended in 250 mL water on ice. Centrifuged again under the same conditions and resuspended in 8.75 mL of cold water and 1.25 mL of 80% glycerol. Again, centrifuged under the same conditions and resuspended in 1 mL of 10% glycerol. 100 µL of aliquots were prepared in eppies and immediately frozen in using liquid nitrogen and long term stored at -80^0C.

3.7.4.3. Electro-transformation

50 µL of electrocompetent cells transferred together with 1-2 µL of pMC3 plasmid into cooled electroporation cuvettes (biozyme) and treated with an electric shock for 4 msec. Subsequently, the cells were transferred to 500 µL LB medium and incubated for 60 mins at 37^0C and then plated on selection plates.

3.7.4.4. Non-denaturation purification of CsgA His-tagged from bacterial supernatant

CsgA protein was purified and incubated with respective plant extracts to see if plant extracts did have some inhibitory effects or not. CsgA His-tagged protein was purified from bacterial supernatant without denaturing the cells as per the protocol followed by the laboratory of Matthew R. Chapman (Zhou et al., 2013) with some modifications as per the suitability of our lab. For 1 L of desired prep, an overnight culture of freshly transformed LSR12/pMC1/pMC3 in 25 mL of antibiotics supplemented LB at 37^o C. This overnight activated culture was used to inoculate 1L of desired antibiotics supplemented LB and incubated with shaking at 37^o C to about 1 OD_{578}. To this 250 µL of 1M IPTG stock was added to induce expression for 45 min at 37^o C. Bacterial supernatant was collected by centrifugation at 10,000 x g for 15 mins at 4^o C and filtered through 0.22 µm polyethersulfone (PES) bottle-top filter.

Purification of protein was performed at 4^oC room. Glass column of 2.5 x 10 cm was packed with 4 mL of Ni-NTA. The bed column was washed with 4-bed volume (BV) of potassium phosphate buffer (50 mM, pH 7.2). The supernatant flowed through the column with the maximum speed. After supernatant flowed through, the column was washed with 10 BV of

phosphate buffer. Proteins were eluted with 2 BV of elution buffer 1 (100 mM imidazole in phosphate buffer) and 5 mL of elution buffer 2 (500 mM imidazole in phosphate buffer) depending on how tightly protein was bound. Reasonable concentrations of protein (concentrations measured at UV 280 at room temperature) fractions were combined and used further for desalting the purified protein samples using the PD10 column. The final elution was done with 2.5 mL phosphate buffer and kept on ice immediately.

3.7.4.5. Biochemical assay: Polymerization of CsgA from purified monomers

This purified protein was further used for biochemical assay. Polymerization of CsgA from monomers was performed onto a non-treated, flat-bottom, non-binding, opaque Greiner 96-well microtiter plate. To 100 µL of freshly prepared purified protein, an optimized concentration of plant extracts was also added. To each well 20 µM of Thioflavin T (ThT) was added and mixed well. The plate was sealed with a transparent sticker and read it at an excitation wavelength to be 438 nm, the emission wavelength 495 nm, and a cut-off filter at 475 nm. The program was set to read the plate every 10 mins at room temperature over 24 hours. Potassium phosphate buffer and untreated purified CsgA were used as controls. Epigallocatechin gallate (EGCG) of 50 µg/mL with CsgA was used as a positive control. Additional control for each plant extract containing phosphate buffer along with an optimized concentration of extracts was also considered.

3.7.4.6. Transmission Electron Microscopy as a control to verify the results of CsgA polymerization assays

Negative staining TEM was performed on curli samples. Samples were applied on Formvar film-coated copper grids having 300-mesh (Electron Microscopy Sciences) for 2 mins. Deionized with water and negatively stained for 90 sec with 2% uranyl acetate. After air-drying microscopy was performed (Reichhardt et al., 2015).

4. Results

4.1. Physicochemical characteristics of plant extracts and their effects on growth medium

The pH of most of the crude plant extracts (both before and after concentration) was acidic showing pH between 4-5; checked with pH strips. Interestingly, *G. mangostana* was found to be highly acidic (pH between 0-1) and *T. chebula* and *C. whighitti* were also strongly acidic; pH ranging between 2-3 (Table 5). Taking into account their acidic nature, further, it has been investigated if these acidic extracts were bringing any change in the pH of the growth medium of bacteria. None of the optimized concentration of plant extract (Table 4) was bringing any change in the final pH of the working LB medium.

All the plant extracts were getting precipitated during the process of concentration (Table 5). However, by re-suspending the completely dried material in 1:1 ratio of water:ethanol most of the precipitation was gone.

Table 5: Physicochemical characteristics of plant extracts and their effects on growth medium

Plant	**pH**		**Precipitation**		**pH of LB before and after addition of extract (3 mL LB + (X) µL extract)**	
	Before concentration	After concentration	Before concentration	After concentration	Before concentration	After concentration
Tinospora cordifolia	~ 5	~ 5	No	Yes	~ 7	~ 7
Cinnamomum zeylanicum	b/w 5-4	b/w 5-4	No	Yes	~ 7	~ 7
Garcinia mangostana	b/w 0-1	b/w 0-1	No	Yes	~ 7	~ 7
Bacopa monnieri	~ 5	~ 5	No	Yes	~ 7	~ 7
Commiphora whighitii	b/w 5-4	~ 3	No	Yes	~ 7	~ 7
Terminalia chebula	~ 3	~ 2	No	Yes	~ 7	~ 7
Glycyrrhiza glabra	~ 5	~ 5	No	Yes	~ 7	~ 7
Fagonia arabica	~ 5	~ 5	No	Yes	~ 7	~ 7

Acorus calamus	b/w 5-4	b/w 5-4	No	Yes	~ 7	~ 7
Aegle marmelos	~ 5	~ 5	No	Yes	~ 7	~ 7

4.2. Growth curves with and without plant extracts

In the presence of plant extracts (ref. Table 4 for optimized concentrations of plant extracts used in the experiments), *E. coli* showed a wide range of growth patterns ranging from no effect to longer lag phase or short exponential phase and unexpectedly higher absorbance at the end of incubation of 24 hrs with respect to the reference strain, W3110 grown in the absence of plant extract. For instance, *T. cordifolia* did not affect the growth of bacteria in liquid culture (Figure 5). On the other hand, *C. zeylanicum* (Figure 5), *C. whighitti* (Figure 6), *G. mangostana* (Figure 5) showed a long lag phase, later attained the same OD as that of control without plant extract after 24 hrs. except in *G. mangostana* where it was lower than the control strain without plant extract. *B. monnieri* (Figure 5), *G. glabra* (Figure 6), *F. arabica* (Figure 6), and *A. marmelos* (Figure 7), showed long exponential phase, and the OD after 24 hr of growth was also higher with respect to control strain, W3110 (without plant extract treatment). In the case of *T. chebula*, the log phase was very short and it tends to move to the stationary growth phase very early. In contrast, *A. calamus* (Figure 7) was showing a short log phase and it entered the stationary phase very early but later after 24 hrs of growth, it attained higher OD than the reference strain. Plant extract in LB medium without plant extracts was also considered as controls.

Irrespective of increase or decrease of values at OD 578 nm, colony-forming units/mL (cfu/mL) were significantly higher with each plant extract. Optical density at 578 nm vs cfu/mL is shown in Figure 8 and real values are shown in Table 6. This is giving a clue that the bacteria in these cases might tend to divide and re-divide rather than adopting the characteristic physiology of the stationary phase which is also associated with biofilm formation.

Table 6 Colony forming unit (cfu/mL) of E. coli (W3110) with and without plant extracts corresponding to their OD-578 nm after 24 hr of growth at 28^{o}C at 200 rpm in water bath

Sr. No.	Sample	OD_{578} after 24 hr	Colony forming unit (cfu)/mL
1	*E. coli* (Without plant extract)	4.2	2.40×10^{9}
2	*Tinospora cordifolia*	4.8	3.80×10^{9}
3	*Cinnamomum zeylanicum*	4.0	5.00×10^{9}

4	*Garcinia mangostana*	3.2	5.00 X 10^9
5	*Bacopa monnieri*	7.4	1.01 X 10^{10}
6	*Commiphora whighitii*	4.3	8.00 X 10^9
7	*Terminalia chebula*	2.8	8.50 X 10^9
8	*Glycyrrhiza glabra*	5.3	1.01 X 10^{10}
9	*Fagonia arabica*	6.0	1.30 X 10^{10}
10	*Acorus calamus*	5.0	1.58 X 10^{10}
11	*Aegle marmelos*	7.4	1.78 X 10^{10}

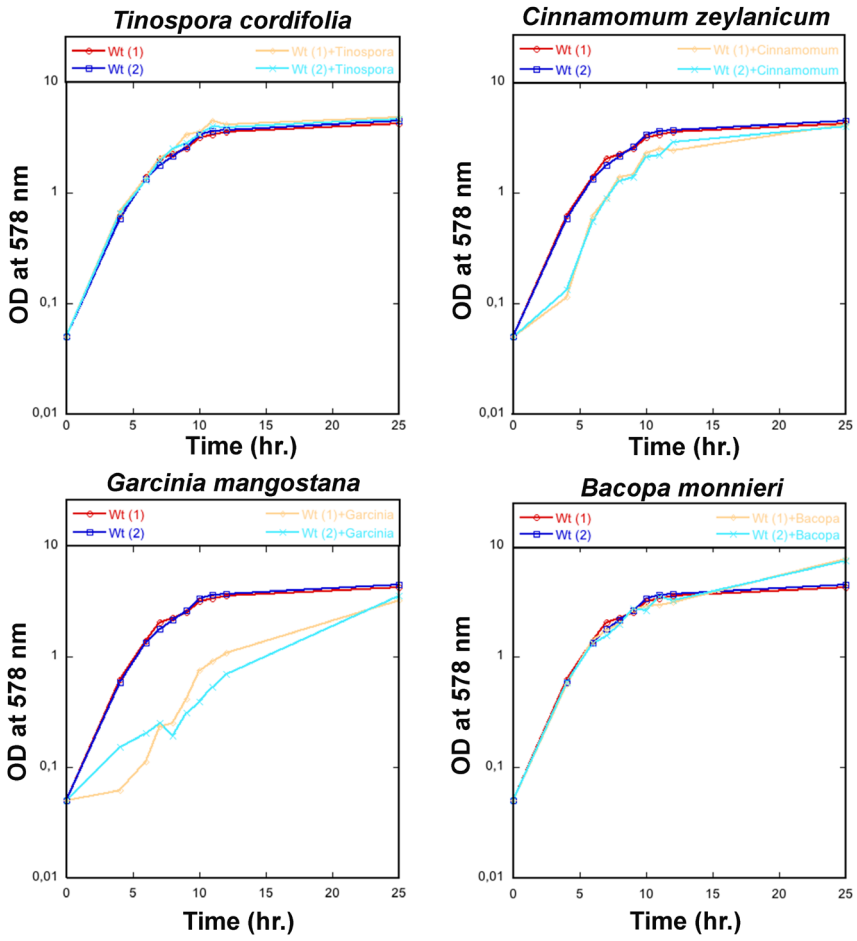


Figure 5*:* Growth curve of E. coli (two biological replicates) in the presence and absence of *T. cordifolia, C. zeylanicum, G. mangostana,* and *B. monnieri*. No effect on growth was observed in the presence *T. cordifolia. C. zeylanicum* and *G. mangostana* delayed exponential phase and *B. monnieri* showed higher OD in comparison to the control after 24 hrs of incubation. The bacterial cultures were incubated at 28 Deg. C at 200rpm in a water bath for 25 hrs. OD at 578 nm of start culture was 0.05. Only plant extract in LB medium was also considered as one of the control and actual OD values of reference strain treated with plant extract were considered

taking LB+plant extract as reference blank. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

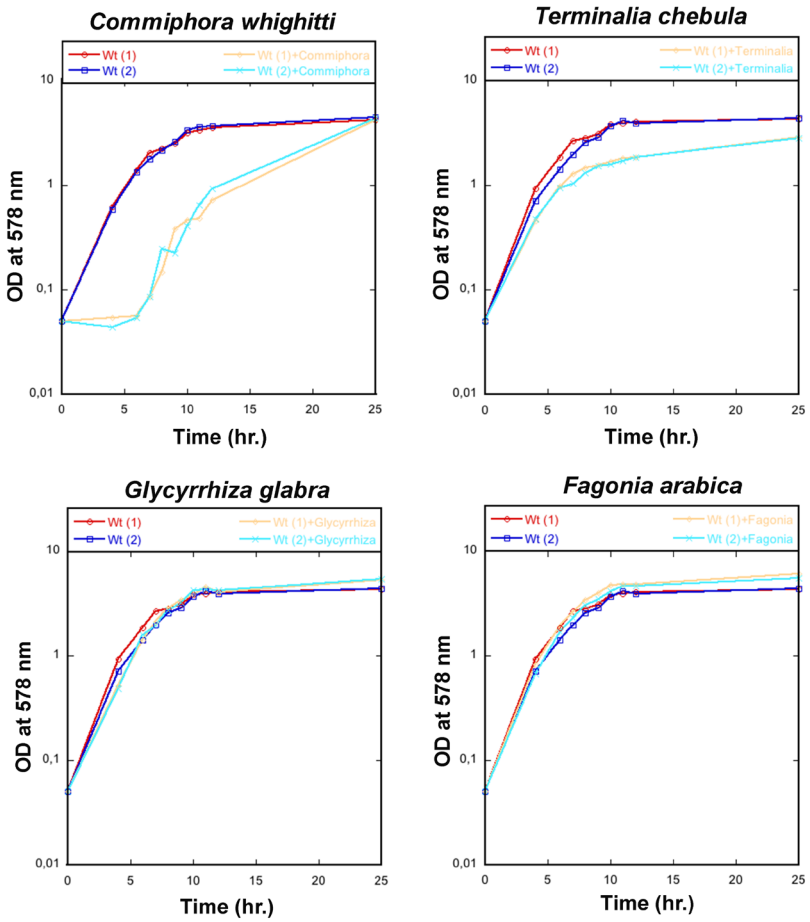


Figure 6*:* Growth curve of *E. coli* (two biological replicates) in the presence and absence of *C. whighitti, T. chebula, G. glabra,* and *F. arabica*. *C. whighitti,* and *T. chebula* showed toxicity and delayed the growth whereas *G. glabra,* and *F. arabica* showed higher OD values. Cultures were incubated at 28 Deg. C at 200rpm in water bath for 25 hrs. OD at 578 nm of start culture was 0.05. Only plant extract in LB medium was also considered as one of the control and actual OD values of reference strain treated with plant extract was considered taking LB+plant extract as reference blank. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

4.3. Optimum concentrations of plant extracts using *E. coli* as a model bacterium

Different concentrations of plant extracts had been used to choose one which has only an anti-biofilm effect without any antimicrobial activity. For screening of anti-biofilm effects of plant extracts, a set of four different strains such as AR3110 (both curli and pEtN-cellulose), W3110 (only curli), AR282 (only pEtN-cellulose), and AR198 (no curli and no pEtN-cellulose) were used showing different colony morphology phenotypic characteristics (explained in section 3.3). Loss or reduction in any distinctive colony morphology such radial ridge in AR3110

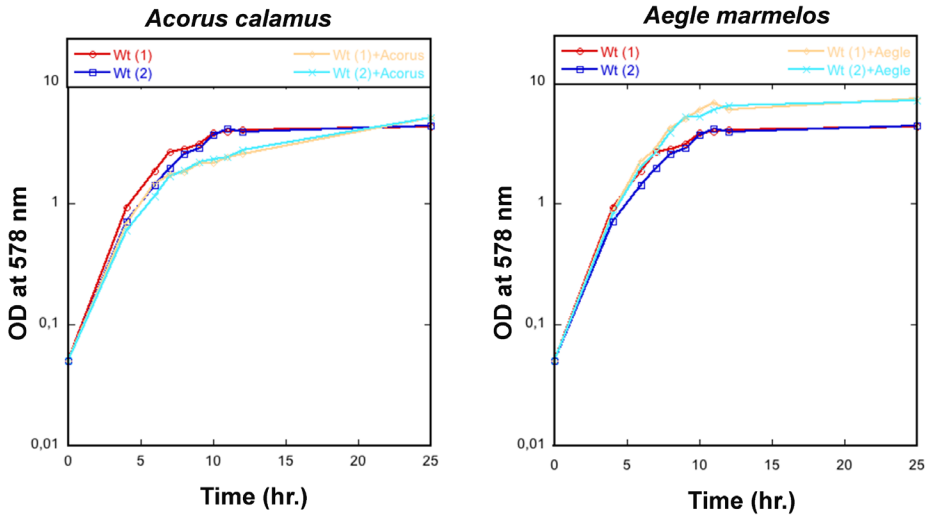


Figure 7*:* Growth curve of *E. coli* (two biological replicates) in the presence and absence of *A. calamus* and *A. marmelos*. Both showed higher OD values at the end of the experiment. Culture were incubated at 28 Deg. C at 200rpm in water bath for 25 hrs. OD at 578 nm of start culture was 0.05. Only plant extract in LB medium is also considered as one of the control and actual OD values of reference strain treated with plant extract were considered taking LB+plant extract as reference blank. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

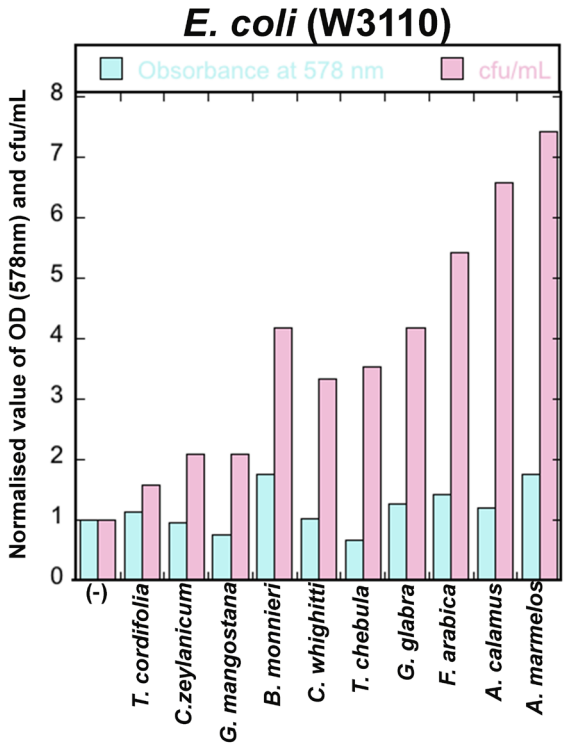


Figure 8*:* Absorbance at 578 nm vs colony forming units (cfu/mL). All the plant extracts treated W3110 strain showed high cfu/mL in comparison to the untreated control strain (no plant extract). Bacterial cultures were incubated at 28 Deg. C at 200rpm in a water bath for 24 hrs. OD at 578 nm of start culture was 0.05. Cfu/mL was calculated by using 3 technical replicates

from the same flask. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

(producing both curli and pEtN-cellulose) and/or concentric rings in W3110 (producing only curli fibers) and/or intertwined wrinkles in AR282 (producing only pEtN-cellulose) and/or reduction or loss of color production in the presence of Congo red was the criteria of analysis of anti-biofilm effects and selection of optimum concentration (Diego O. Serra et al., 2013). AR198 (no curli and pEtN-cellulose) was an important strain to check the antimicrobial effect in plant extracts if any with a reduction in the diameter of the colony. In most of the cases, the effective anti-biofilm concentration of plant extracts was 60 µL per 3 mL of LB without salt and supplemented with Congo red (CR) (Figure 9 A, B, C, D, E, F, G, H, I and J). In the case of *C. zeylanicum* 20 µL per 3 mL medium, 10 µL in *G. mangostana* and *T. chebula* per 3 mL of the medium had been selected for further studies as beyond these concentrations anti-biofilm effect was combining with antimicrobial effect as the size of the colony of AR198 (no curli and no pEtN-cellulose) decreases with increasing concentration of plant extracts. It was clear from the data that the anti-biofilm effect was dose-dependent. Lower concentrations of plant extracts in the case of *T. chebula, G. mangostana,* and *C. zeylanicum* were effective as anti-biofilm in contrast to higher concentrations where the effects were antimicrobial.

The first step to screen the anti-biofilm effects of plant extracts in this study was by performing macrocolony on agar plates. All the 10 plant extracts used in this study showed highly effective anti-biofilm effects against *E. coli* K-12 on macrocolonies. These plant extracts had also been studied on macrocolonies of *B. subtilis* and various other pathogenic bacteria such as EAEC, UPEC, *P. aeruginosa,* and *S. aureus*. In the case of *E. coli*, a set of 4 mutant reference strains AR3110 (both curli and pEtN-cellulose), W3110 (only curli), AR282 (only pEtN-cellulose), and AR198 (no pEtN-cellulose and no curli) (details of the strains and their morphological phenotypes are explained in previous sections) were used to screen the anti-biofilm effects at the phenotypic level.

As mentioned earlier, loss or reduction of distinctive macrocolony morphologies was a criterion to screen the anti-biofilm effects of plant extracts in the case of *E. coli*. Anti-biofilm effects of *T. cordifolia* and *C. zeylanicum* were shown in Fig 9 (A&B). With the extracts of *T. cordofolia*, significant anti-biofilm results was seen on both curli and pEtN-cellulose as characteristic ridges in AR3110 (both curli and pEtN-cellulose), concentric rings in W3110 (only curli) and

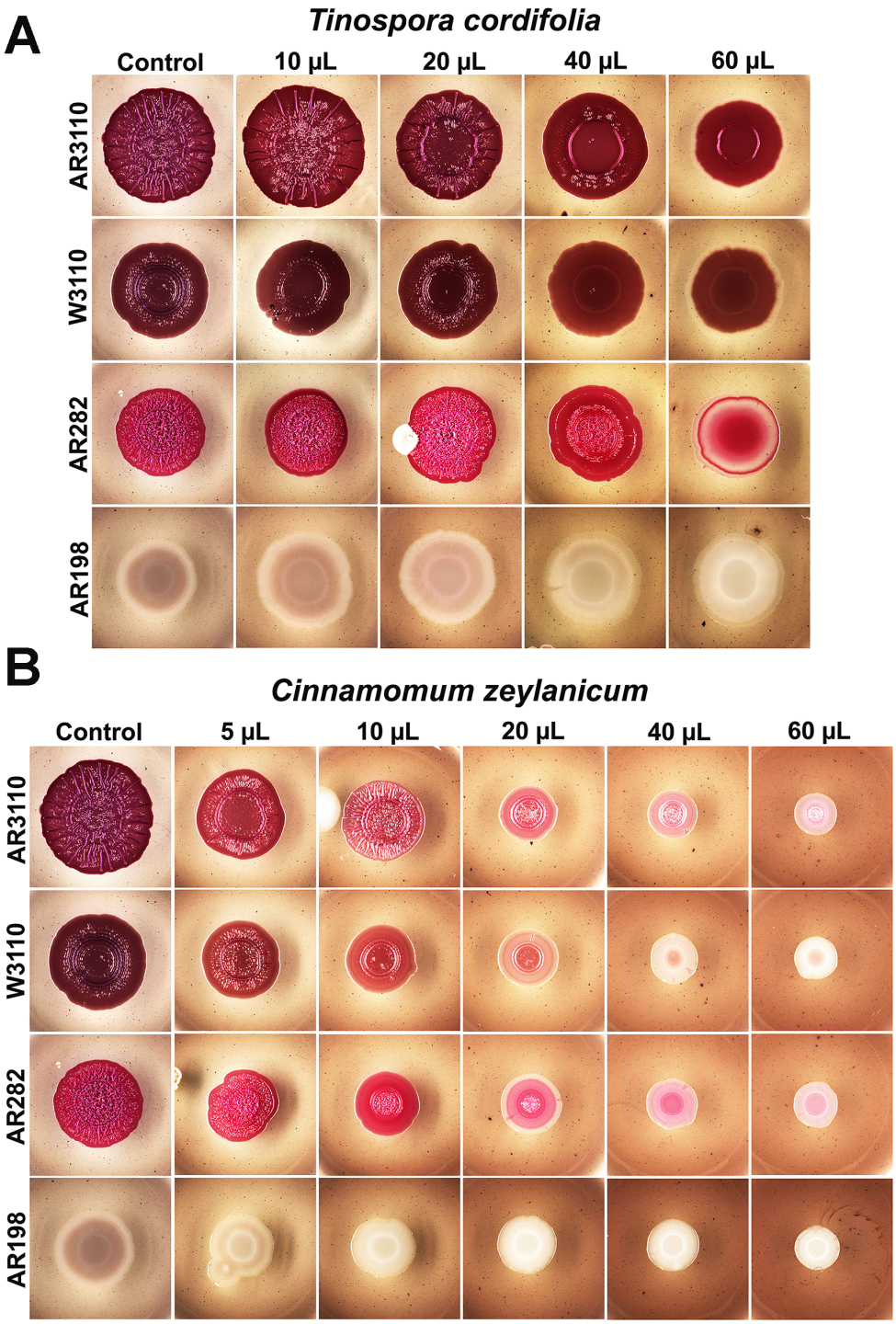


Figure 9 (A&B): Optimization of concentration of plant extracts for anti-biofilm effects (A) *T. cordifolia* and (B) *C. zeylanicum* using macrocolony morphology of AR3110 (both curli and cellulose), W3110 (only curli), AR282 (only cellulose) and AR198 (no curli and no cellulose). The optimized concentration for *T. cordifolia* was 60 µL and *C. zeylanicum* was 20 µL. Both the plant extracts have a significant effect on both curli and cellulose. *C. zeylanicum* showed growth inhibition beyond 20 µL. All the strains were grown at 28 deg. C for 5 days on LB without salt supplemented with Congo red.

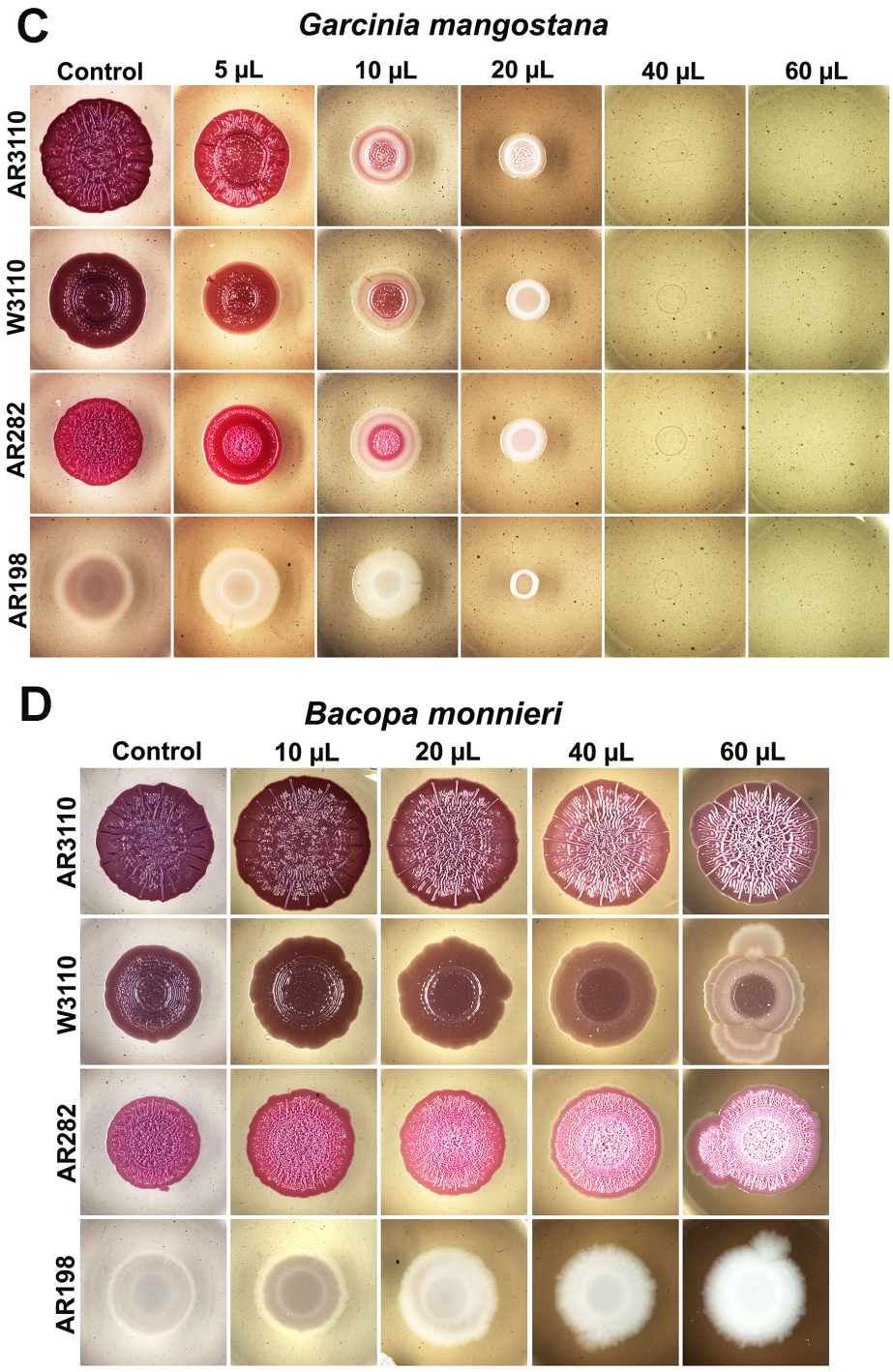


Figure 9 (C&D): Optimization of concentration of plant extracts for anti-biofilm effects (C) *G. mangostana* and (D) *B. monnieri.* The optimized concentration for *G. mangostana* was 10 µL and *B. monnieri* was 60 µL. *G. mangostana* has a significant effect on both curli and cellulose whereas *B. monnieri* greatly reduced curli formation without any effect on cellulose. *G. mangostana* showed strong growth inhibition at 20 µL and onwards. (Ref. Fig. 9 A&B for experimental details).

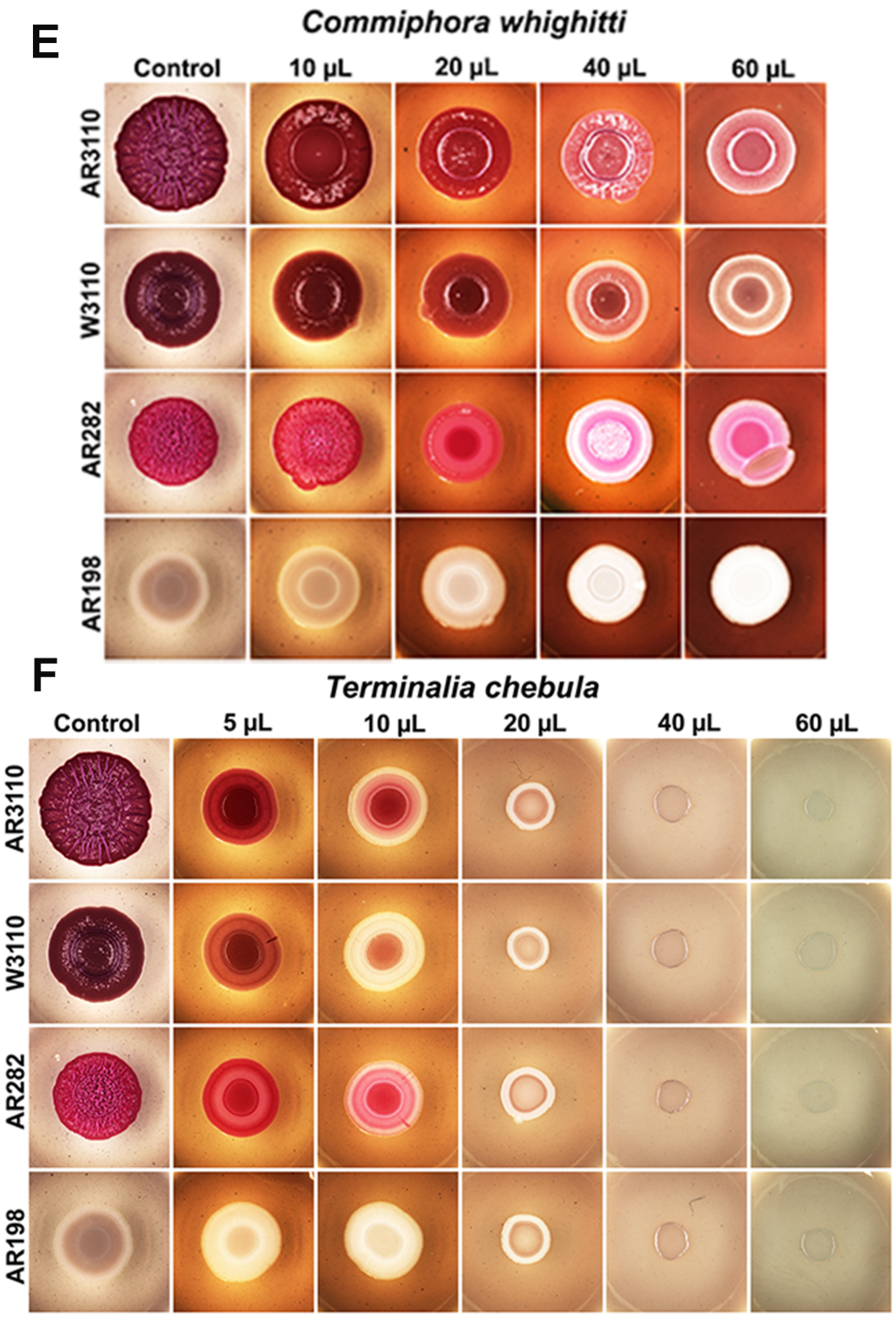


Figure 9 (E&F): Optimization of concentration of plant extracts for anti-biofilm effects (E) *C. whighitti* and (F) *T. chebula* using macrocolony morphology. The optimized concentration for *C. whighitti* was 60 µL and *T. chebula* was 10 µL. Both the plant extracts have a significant effect on both curli and cellulose. At 20 µL and onwards *T. chebula* inhibited the growth. *T. chebula* also showed complete discoloration in case W3110 signifying strong effect on curli. (Ref. Fig. 9 A&B for experimental details).

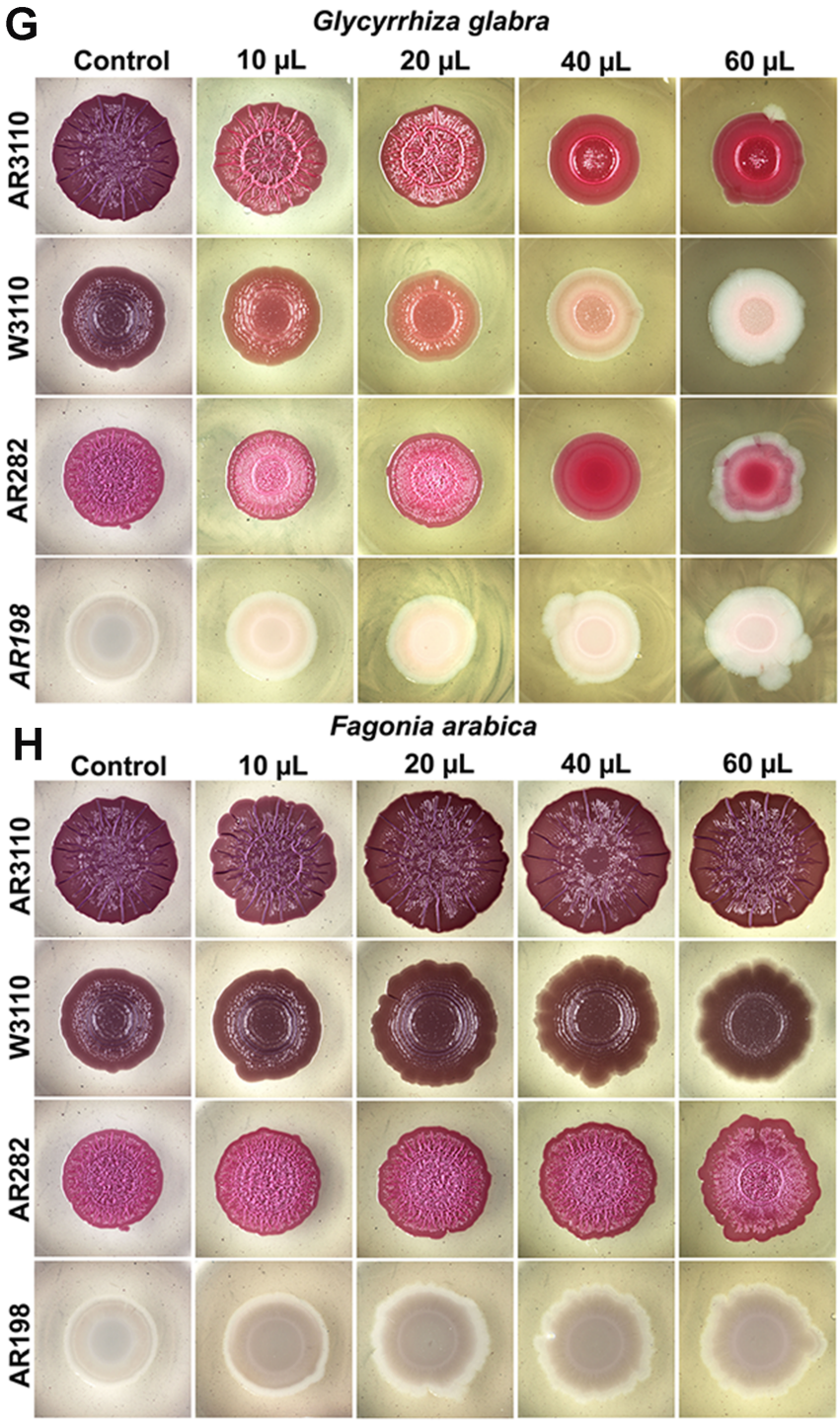


Figure 9 (G&H): Optimization of concentration of plant extracts for anti-biofilm effects (G) *G. glabra* and (H) *F. arabica* using macrocolony morphology. The optimized concentrations for *G. glabra* and *F. arabica* were 60 µL. *G. glabra* had a significant effect on both curli and cellulose whereas *F. arabica* greatly reduced curli formation without any effect on cellulose. Complete discoloration was observed with *G. glabra* with W3110 signifying a very strong effect on curli. (Ref. Fig. 9 A&B for experimental details).

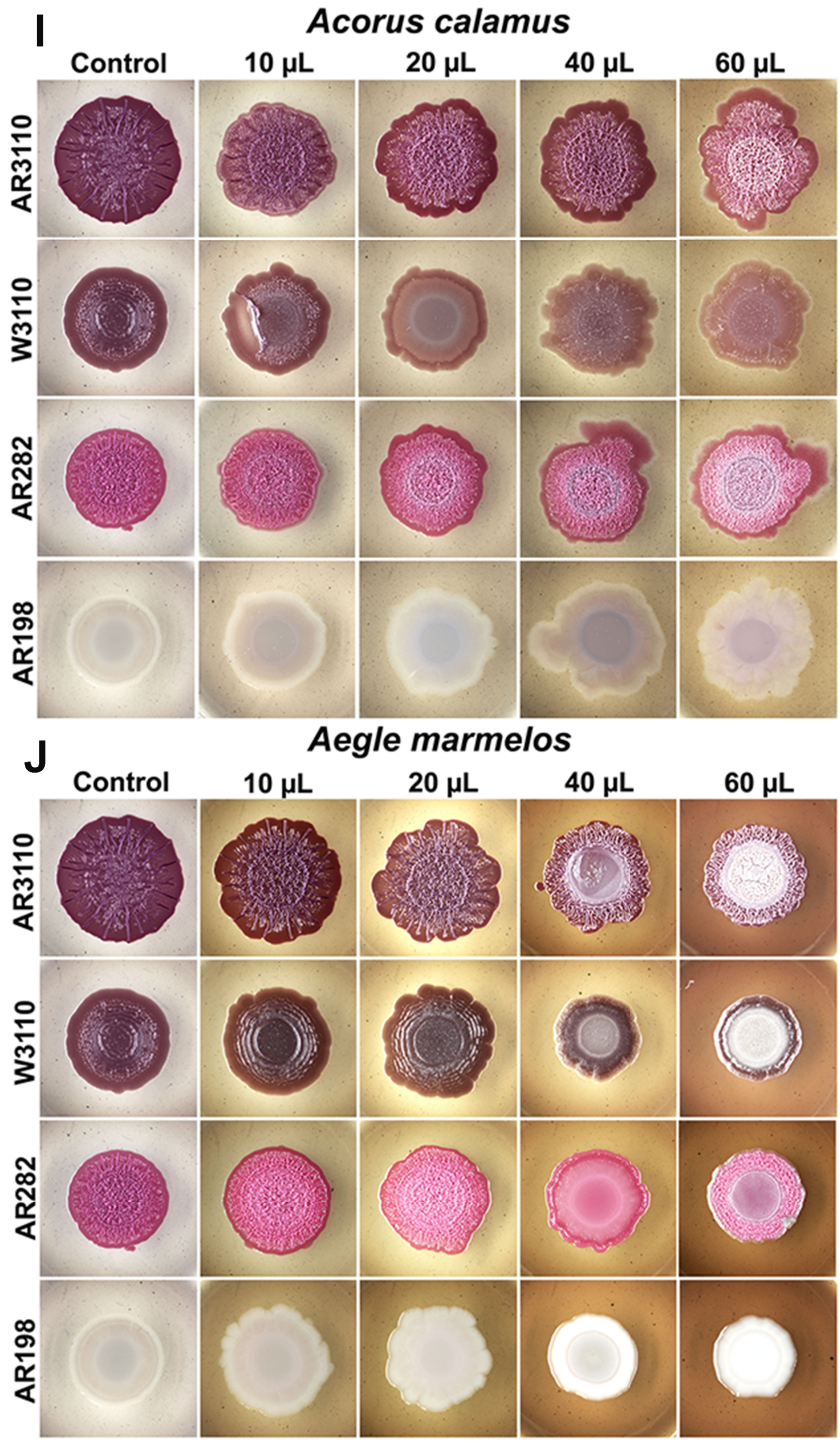


Figure 9 (I&J): Optimization of concentration of plant extracts for anti-biofilm effects (I) *A. calamus* and (J) *A. marmelos* using microcolony. Optimized concentrations for *A. calamus* and *A. marmelos* were 60 µL. *A. calamus* had a significant effect on only curli whereas *A. marmelos* showing very interesting morphology, white at the center, and colored at the edges but having effects at both curli and cellulose. (Ref. Fig. 9 A&B for experimental details).

intertwined wrinkles in AR282 (only pEtN-cellulose) were absent after incubation of 5 days. The characteristic color of all the above said 3 strains was retained. *C. zeylanicum* and *G.*

mangostana (Fig. 9 B &C) showed different zones with color differentiation in the colonies which supports the evidence that within the biofilm, heterogeneity (physiological differentiation) did exist. Heterogeneity within the biofilm has already been documented by various other studies also (Klauck et al., 2018; D. O. Serra & Hengge, 2014; Stewart & Franklin, 2008).

Production of characteristic color and distorted structures in the middle of the colony in case *G. mangostana* was suggesting either delay or suppression of components of the biofilm formation. Interestingly, in the case of *B. monnieri* (Fig 9 D) highly strong effect was observed in the case of curli with no effect on pEtN-cellulose production after the incubation of 5 days at 28 deg. C. In the case of AR3110, surprisingly, more pronounced ridges were produced.

Figure 9 (E&F) shows strong anti-biofilm effects of *C. whighitti* and *T. chebula* on both curli and pEtN-cellulose production. The strong discoloration was also observed in W3110 (only curli) with the extract of *T. chebula*. Complete loss of characteristic color in the case of W3110 (only curli) was observed with extract of *G. glabra* in contrast to *F. arabica* (Fig. 9 G&H). Interestingly, both *F. arabica* (Fig. 9 H), *B. monnieri* (Fig.9 D), and *A. calamus* (Fig. 9 I) showed very strong effects on curli production with no effect on pEtN-cellulose. *F. arabica* produced spreading colonies of AR3110 in comparison to control grown without plant extract. The most interesting observation was made with *A. marmelos* (Fig 9 J) as a colored ring developed at the edges of all three strains, AR3110, W3110, and AR282 with white color in between. Some wrinkling in the case of AR3110 and AR282 was associated with colored rings suggesting that these could be because of pEtN-cellulose production.

4.4. Optimum medium and temperature for the screening of the anti-biofilm effect of plant extracts in the case of *Staphylococcus aureus*

The conventional biofilm growth medium (TSB supplemented with 3% NaCl and 0.5% glucose) for *S. aureus* (Cassat et al., 2007) was tested along with 100 mM of $MgCl_2$ (Wermser & Lopez, 2018) in contrast to TSB only with 100 mM of $MgCl_2$ (Wermser & Lopez, 2018). The wrinkled colony produces at 37 deg. C with TSB only with 100 mM of $MgCl_2$. In addition to this, TSB with 100 mM of $MgCl_2$ with and without Congo red had also been tested. Tryptic Soya Agar (TSA) supplemented with 100 mM of $MgCl_2$ and Congo red was best suited to produce the wrinkled biofilm macrocolonies of *Staphylococcus aureus* (Figure 10 (A)). Congo red further enhanced wrinkling. Biofilm producing wrinkled colonies was temperature-sensitive too. They wrinkled only at 37 deg. C in contrast to 28 deg. C.

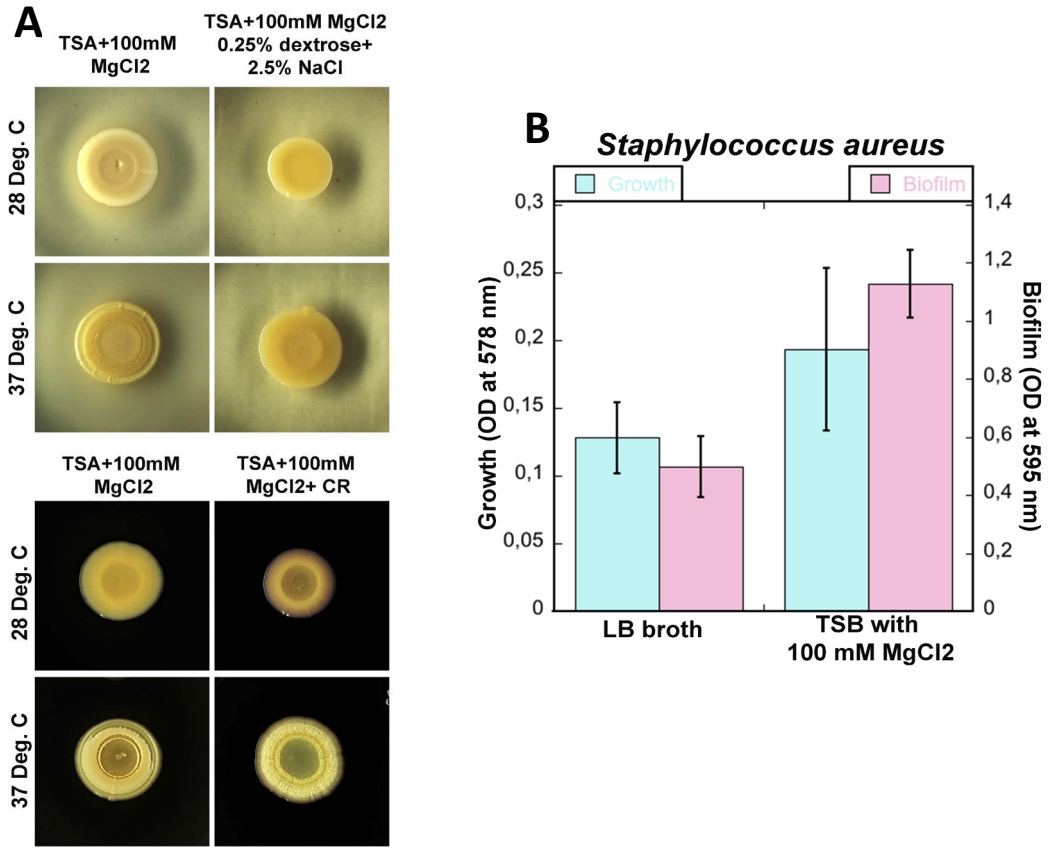


Figure 10 (A) Optimization of media for *S. aureus* macrocolonies. At 37 deg. C on Tryptic Soya Agar with 100 mM of $MgCl_2$, wrinkled colonies were produced. With supplementation of Congo red in the same medium, enhanced wrinkling was observed. **(B)** Both submerged biofilm and biomass increase, when grown in Tryptic Soya Broth with 100 mM of MgCl2 on fibronectin, coated 96 well plates in contrast to LB medium. All the data sets are mean ± standard deviation of 9 technical replicates.

It has already been well documented that with the addition of human plasma in growth medium or coated onto 96 well plate, can influence the gene expression favoring the biofilm formation in the case of *Staphylococcus aureus* (Aly & Levit, 1987; Cardile et al., 2014; Herrmann et al., 1988). Fibronectin is one of the major components involved in the attachment of *S. aureus* on the surfaces (Herrmann et al., 1988). For optimization of submerged biofilm of *S. aureus*, 96 well plate coated with human fibronectin was used for the development of submerged biofilms. Figure 10 (B) showed that TSB supplemented with 100 mM of $MgCl_2$ enhanced growth and submerged biofilm in comparison to LB broth when grown on fibronectin-coated 96 well plate.

4.5. Effect of plant extracts on macrocolony biofilms

In the case of other tested microorganisms such as *B. subtilis* and various other pathogenic bacteria such as EAEC, UPEC, and *P. aeruginosa,* there was no such reference mutant strains to screen anti-biofilm effects. Only by investigating the change in color, change in macrocolony

morphology phenotype with respect to the strains grown in the absence of plant extracts some observations had been made. Data somehow clearly showed that there maybe some physiological changes in the presence of plant extracts and that could be biofilm suppressing or enhancing or any other unknown physiological behavior. The following are the detailed effects of plant extracts on different bacterial species.

4.5.1. Enteroaggregative *E. coli* (EAEC)

Biofilms of EAEC are not associated with the production of curli fibers or pEtN-cellulose (Croxen & Finlay, 2010). Still, characteristic reddish color and ridges were observed at 28 deg. C in control (Fig. 11). At 37 deg. C, a dark reddish color colony with no distinctive structures developed. Colonies were flat having a colorless zone in the middle. Diverse colony morphologies of Enteroaggregative *E. coli* were observed with the treatment of different plant extracts. Because of the lack of reference mutant strains to screen anti-biofilm effect, it was difficult to say if these plant extracts do have any anti-biofilm effects on these macrocolonies. It was still interesting to see how one bacterial strain can show a wide range of colony morphologies in the presence and absence of different plant extracts.

4.5.2. Uropathogenic *E.coli* (UPEC)

Whitish, circular, and raised colonies were observed at 28 deg. in contrast to colonies at 37 deg. C (Figure 11) which were reddish in color with a whitish zone in the middle and edges. There was nothing much interesting observed with the treatment of plant extracts except *B. monnieri*, *G. glabra,* and *A. marmelos.*

With *B. monnieri*, highly filamentous colonies were produced at both 28 deg. C and 37 deg. C. with the production of a pinkish color ring which was seen only in case at 37 deg. C. In the case of *G. glabra, a* similar filamentous colony with a pinkish zone in the middle was observed. With *A. marmelos,* small mucoid circular colonies having undulate margins developed.

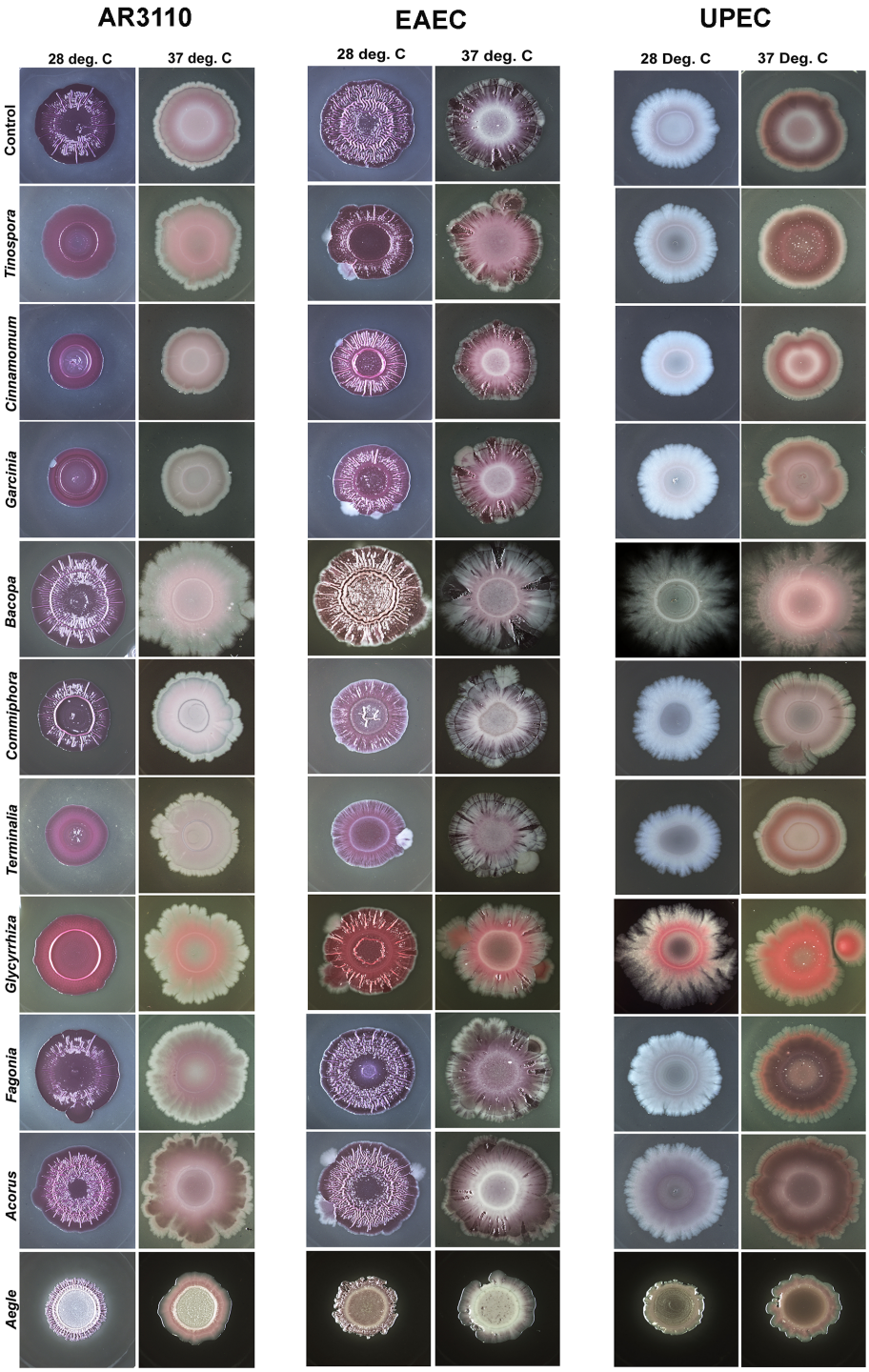


Figure 11: Anti-biofilm effect of plant extracts on pathogenic *E. coli* (EAEC and UPEC) in comparison to lab strain AR3110. No major effects were observed both in case of EAEC and UPEC. *B. monnieri, G. glabra* and *A. marmelos* has significant effects on colony morphology both in the case of EAEC and UPEC. All the strains were grown both at 28 deg. C and 37 deg. C for 5 days on LB without salt supplemented with Congo red in the presence and absence of plant extracts. Images were captured using stereomicroscopy. Diverse range of phenotypic colony morphology was observed. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

4.5.3. *Pseudomonas aeruginosa*

Figures 12 showed colony morphologies of *Pseudomonas aeruginosa* at and 28 deg. C after 5 days of incubation. *P. aeruginosa* develops morphologically so diverse colonies. At 28 deg. C, it produced circular, pinkish to purplish colony whereas at 37 deg. C, it produced a colony showing different zones of colors ranging from bluish to greenish shades. *P. aeruginosa* is known to produce many pigments such as blue pigment is pyocyanin, yellow-green is pyoveridin, red is pyorubin, and brown is pyomelanin and out these pyocyanin has various biological applications such as antifungal activity (DeBritto et al., 2020; Kerr et al., 1999).

Most interestingly, these different pigments producing colonies were observed when treated with different plant extracts. With *T. cordifolia* and *C. whighitti* there was not much difference in colony morphology that can be observed at both temperatures. With *C. zeylanicum*, *G. mangostana* and *T. chebula* brown pigment has been produced which was much more pronounced in the case of *G. glabra* at 37 deg. C. In the case of B. *monnieri*, *F. arabica,* and *Acorus calamus* at 37 deg. C., large spreading colonies almost covering the 35-mm plate were produced that were further producing dense greenish pigment, phenazines.

At 28 deg. C. some of the plant extracts supported the wrinkling of macrocolonies such which was pronounced in the case of *T. chebula*, and kind of suppressed wrinkling was produced in the case of *C. zeylenicum, G. mangostana, C. whighitti and A. marmelos* suggesting an increase in production of extracellular matrix (Dietrich et al., 2013). Colour of the colonies was pinkish to purplish. In the case of *G. glabra* and *F. arabica* very large outward spreading colonies were observed almost reaching to the edges of 35 mm plate. (NOTE: all the images are taken at the same magnification under stereomicroscope but image crop size on Photoshop software is different. For all the images with red boundaries, the image crop size is 1600 px X 1600 px. Rest all have crop size 1200 px X 1200 px).

4.5.4 *Staphylococcus aureus*

Under optimized medium and conditions, *S. aureus* produced circular and wrinkled colonies (Figure 12). With the treatment of plant extracts, wrinkling structures were not visible after the incubation of 5 days. Rather, with some plant extracts, red color colonies were produced. With *B. monnieri*, a large colony with red edges were produced. This red color could be some unknown extracellular component of the bacteria which either itself was colored or reacting to

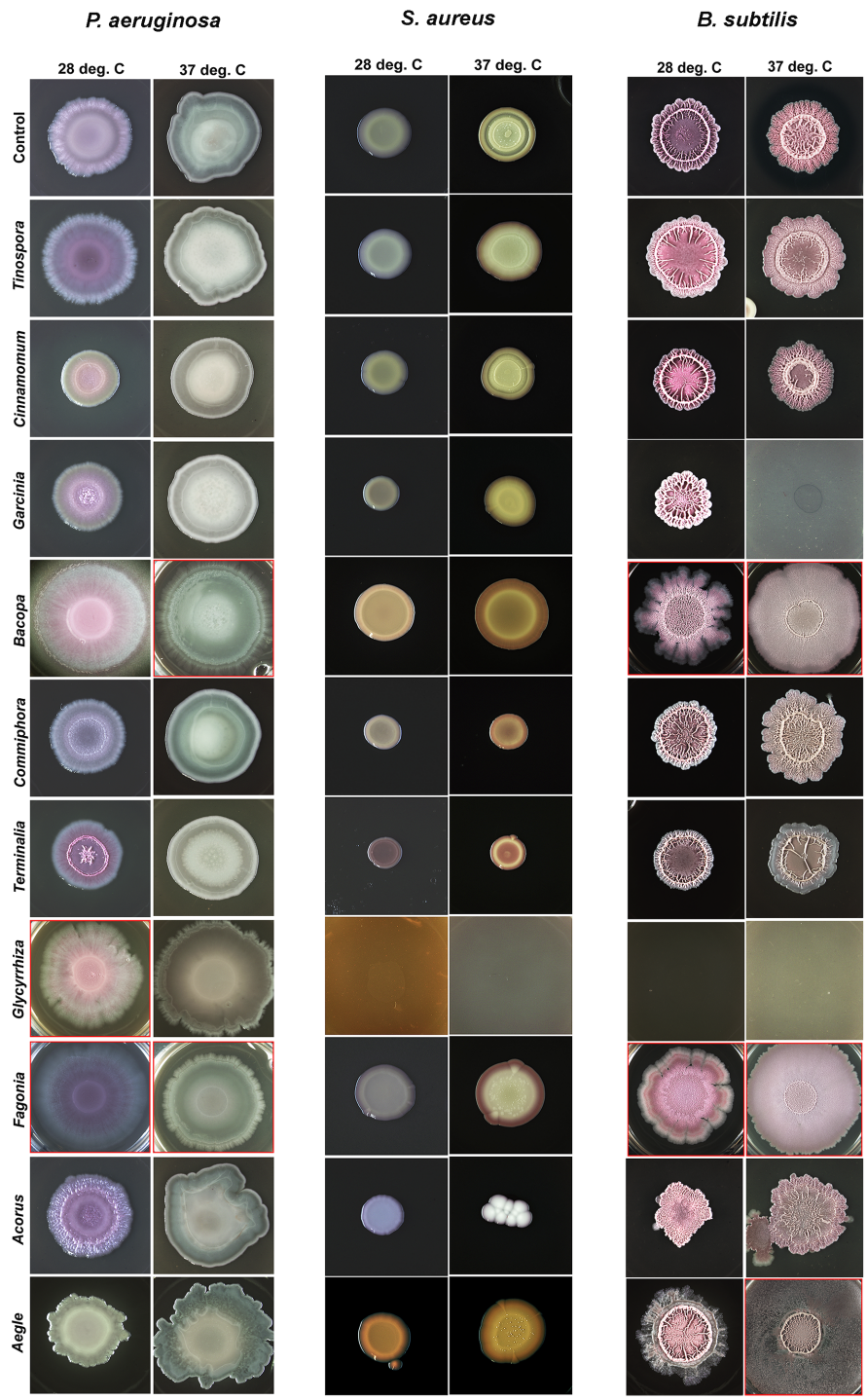


Figure 12: Broad range anti-biofilm effect of plant extracts on Gram negative (*P. aeruginosa*) and Gram positive (*S. aureus* and *B. subtilis*). *P. aeruginosa* developed morphologically so diverse colonies. In the case of *B. monnieri*, *F. arabica,* and *A. calamus* at 37 deg. C., large spreading colonies of *P. aeruginosa* almost covering the 35-mm plate were produced that were further producing dense greenish pigment, phenazines. Under optimized medium and conditions, *S. aureus* produced circular and wrinkled colonies. With the treatment of plant extracts, wrinkling structures were not visible after the incubation of 5 days. A wide range of colony morphologies were observed in the presence of all the plant extracts in case of *B. subtilis*. At 37 deg. C, in the presence of *A. marmelos* unique mesh-like network, was observed around the colony. With *B. monnieri* and *F. arabica*, interestingly, colonies were spreading towards

the edges of the 35-mm petri plate. All the strains were grown both at 28 deg. C and 37 deg. C for 5 days on LB without salt supplemented with Congo red in the presence and absence of plant extracts. Images were captured using stereomicroscopy. Diverse range of phenotypic colony morphology was observed. All the images were captured on same magnification. Crop size of the images on Photoshop software was 1200 px X 1200 px. Crop size of the images with red border were 1600 px X 1600 px. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

Congo red present in the medium. With *C. whighitti* and *T. chebula* size of the colony was smaller with reference to the control with distinctive red color production. *S. aureus* growth was completely inhibited with the *G. glabra*. With *F. arabica*, circular colonies were produced with a reddish color edge. Most interestingly with *A. calamus*, very unique colony morphology was produced. It was whitish and looks like a combination of different circular mucoid sub-colonies.

4.5.5 *Bacillus subtilis*

As mentioned earlier, there were no such reference mutant strains of *B. subtilis* (as we had in the case of *E. coli*) were there to screen the anti-biofilm effect of plant extracts. Interestingly, a wide range of colony morphologies were observed in the presence of all the plant extracts suggesting that there must be some strong alterations at their physiological level (Figure 12). *G. mangostana* at 37 deg. C was acting as antimicrobial against *B. subtilis*. A similar antimicrobial effect was seen with *G. glabra* at both temperatures. With *B. monnieri* and *F. arabica*, interestingly, colonies were spreading towards the edges of the 35-mm petri plate. Color differentiation and colony morphological differentiation within the same colony further suggesting the morphological heterogeneity under the same chemical and physiological conditions at both 28 deg. C and 37 deg. C. In the presence of *A. marmelos,* unique mesh-like network, was observed around the colony. Most recently it has been found that in the case of *B. subtilis* biofilms TasA induces expression of flagellar genes in biofilms and thus creating a subset of the community that expresses motility in sessile biofilms (Steinberg et al., 2020). It could be possible that in the presence of certain plant extracts motility genes were overexpressed within the biofilm and allowing them to spread away from the macrocolony for several unknown reasons. It could be a strategy to find a new niche during the developmental stages of the biofilm which was earlier considered to happen in the late phase of the biofilm when the dispersion is commenced (Vlamakis et al., 2013) or just a way to respond to unknown compounds which might be surfactant or toxin for them (Steinberg et al., 2020) which could be there in plant extract too.

4.6 Effect of plant extracts on submerged biofilms

After screening the anti-biofilm activity of plant extracts on macrocolonies of bacteria, these plant extracts were also subjected to evaluate their effects on submerged biofilms on the same series of bacteria. Bacteria can form biofilms almost anywhere on solid surfaces or liquid environments (Donlan, 2002). It has also been concluded in various studies that the composition of extracellular matrix can vary from species to species and the surrounding conditions (Besharova, Suchanek, Hartmann, Drescher, & Sourjik, 2016; O'Gara, 2007). To see the anti-biofilm effectivity of plant extracts on submerged biofilm, modified classical 96-well microtiter dish biofilm assay was used explained by George A O'Toole in 2011 (G. A. O'Toole, 2011) was used. Natural plant products are already considered to be best suitable for the control of biofilm formation (J. H. Lee et al., 2013).

4.6.1 *E. coli*

Normalized values of growth (OD at 578 nm) and crystal violet (CV) assay (these data sets were means ± standard deviations of crystal violet assay of 3 technical replicates; experiment repeated 3 times) to evaluate biofilm formation were plotted against different bacterial strains untreated and treated with plant extracts. AR3110 (both curli and pEtN-cellulose) was used as model bacteria to study anti-biofilm effect of plant extracts on non-pathogenic *E. coli* (Figure 13 A). None of the plant extracts showed any drastic effect on growth except *G. glabra* which promoted the growth rate in microtiter plate. *T. cordifolia, B. monnieri, G. glabra, F. arabica,* and *A. marmelos* significantly reduced biofilm formation. These plant extracts showed strong effects on macrocolonies as well. These could be strong candidates to be selected for further studies.

Figure 14 was the 96 well plate where X is uninoculated control, Y was AR3110 without plant extract. Each numerical value represents plant extract and A and B in each set was only plant extract in LB medium and plant extract in LB medium inoculated with AR3110 respectively. 1 represents *T. cordifolia* and it was very clear in the well 1(B) that biofilm gets reduced to a great extent. 2 represents *C. zeylenicum* 2 (B) wells represents no reduction in biofilm formation. 3 represents *G. mangostana* and 3 (B) shows enhanced biofilm formation. 4

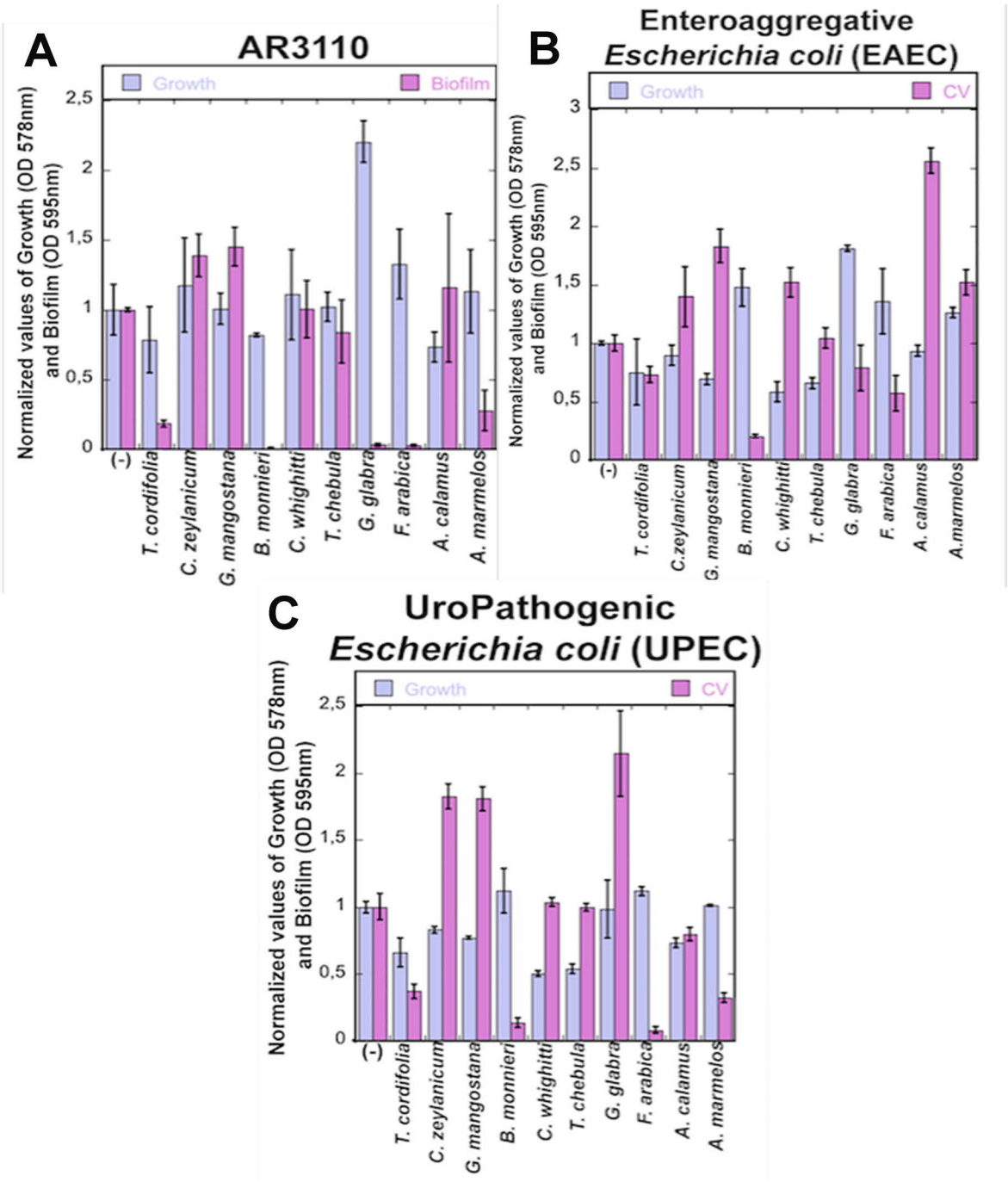


Figure 13 Anti-biofilm effect of plant extracts on submerged biofilm of *E. coli* K-12 and its pathogenic strains. (A) AR3110 *E. coli* K-12, (B) EAEC, and (C) UPEC. *G. glabra* significantly reduced the biofilm of AR3110 without showing any effect on biofilm formation, in contrast, promoted biofilm of UPEC. This shows the antibiofilm effect is strain specific and signifies the diversity of composition of matrix within same species. Respective strains were grown on LB medium in 96 well plate both with and without plant extracts and incubated at 37 deg. C for 24 hrs without shaking. Unincoulated LB medium and plant extracts in LB medium without bacterial inoculation were used as control. Bacterial biomass and absorbance of crystal violet were calculated at 578 nm and 595 nm respectively. Normalized values were plotted with respect to respected conditions. All the data sets are mean ± standard deviation of 3 technical replicates; experiment was repeated 3 times. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

represents *B. monnieri* and 4 (B) showed complete inhibition of biofilm formation. 5 represents *C. whighitti* and 5 (B) depicts enhanced biofilm formation. 6 represents *T. chebula* and 6 (B) showed that it promoted biofilm formation. 7 represents *G. glabra* and 7 (B) showed complete inhibition of biofilm formation. 8 represents *F. arabica* and 8 (B) indicating total inhibition of biofilm formation. 9 represents *A. calamus* and 9 (B) had no effect on biofilm formation and 10 represents *A. marmelos* and 10 (B) showed it suppressed biofilm to a greater extent.

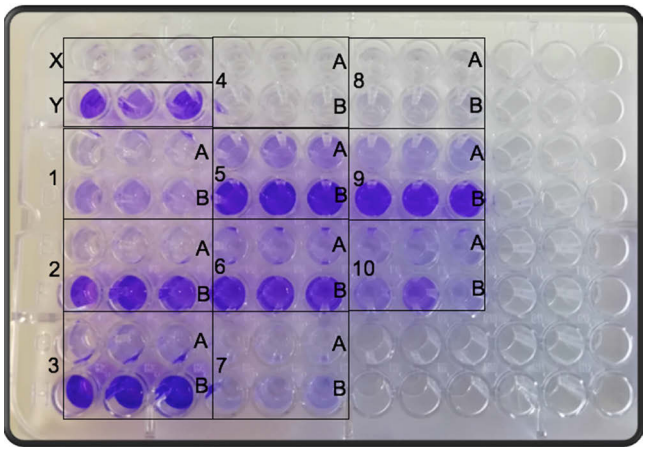


Figure 14: Crystal violet assay of AR3110 on 96 well plate. X is uninoculated control, Y is AR3110 without plant extract. Each numerical value represents plant extract and A and B in each set is "only plant extract in LB medium" and "plant extract in LB medium inoculated with AR3110" respectively. 1 represents *T. cordifolia*; 2 represents *C. zeylenicum*; 3 represents *G. mangostana*; 4 represents *B. monnieri*; 5 represents *C. whighitti*; 6 represents *T. chebula*; 7 represents *G. glabra*; 8 represents *F. Arabica*; 9 represents *A. calamus*; 10 represents *A. marmelos*. Experiment was performed using triplicates. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

4.6.2 Enteroaggregative *E. coli* (EAEC)

In the case of Enteroaggregative *E. coli*, all the plant extracts supported growth (Figure 13 B). Only *B. monnieri* reduced the biofilm significantly. *B. monnieri* was the strongest candidate among all other plant extracts showing significant effects in pathogenic *E. coli* as none other showed effective results in this case.

4.6.3 Uropathogenic *E.coli* (UPEC)

Although *C. whighitti* and *T. chebula* reduced the growth of Uropathogenic *E. coli* (UPEC) to a certain extent yet they did not show any reduction in biofilm formation (Figure 13 C). On the other hand, all other plant extracts did not have any significant effect on growth. *T. cordifolia, B. monnieri*, *F. arabica,* and *A. marmelos* significantly reduced biofilm formation.

4.6.4 *Pseudomonas aeruginosa*

B. monnieri and *A. marmelos* significantly inhibiting biofilm formation without having any effect on the growth of bacteria (Figure 15).

4.6.5 *Staphylococcus aureus*

None of the plant extracts showed significant reduction in biofilm formation in the case of *S. aureus* submerged biofilm. Rather many plant extracts did not support the biomass (Fig. 16).

4.6.6 *Bacillus subtilis*

Surprisingly, most of the plant extracts did not support the growth of *B. subtilis* in microtiter dish plates (Fig. 16) except *C. zeylanicum, B. monnieri,* and *A. marmelos*. Conversely, they grew all well on agar plates except *G. glabra* which did not support any growth. Both *B. monnieri* and *A. marmelos* promoted growth and completely inhibited biofilm formation. Both *B. monnieri* and *A. marmelos* were effectively inhibiting biofilm formation in both Gram-positive. *B. subtilis* and Gram-negative *E. coli* as model organisms. And they both significantly reduced the biofilm on macrocolonies in the case of *E. coli*.

It could be concluded from the submerged biofilm results that *B. monnieri* was the most promising candidate which inhibited biofilm formation in all the bacterial strains except *S. aureus*.

4.7 Effects of plant extracts on biofilm-related gene expression in *E. coli*:

After studying the generalized effects of plant extracts on macrocolonies and submerged biofilm, the next goal of this project was to study the molecular mechanism of the plant extracts to inhibit the biofilm formation.

Getting inspired with the research design of Serra O. Diego with his co-workers (D. O. Serra et al., 2016), the design of this study was formulated to study the mechanism of action with lots of modifications and current research updates. To study the potentially altered expression of biofilm-related genes (if any) by plant extracts, couple of W3110 derivatives carrying single-copy *lacZ* fusions in target gene such as *csgB,* or in *dgcC* or other tested targets genes (Table 2) were grown on LB medium without salt in the absence or presence of plant extracts at 28 deg. C and tested further using beta-galactosidase enzyme assay, expression level were checked by analyzing the enzyme activity of beta-galactosidase. Only plant extracts without any bacterial inoculation were also considered as a control in these experiments.

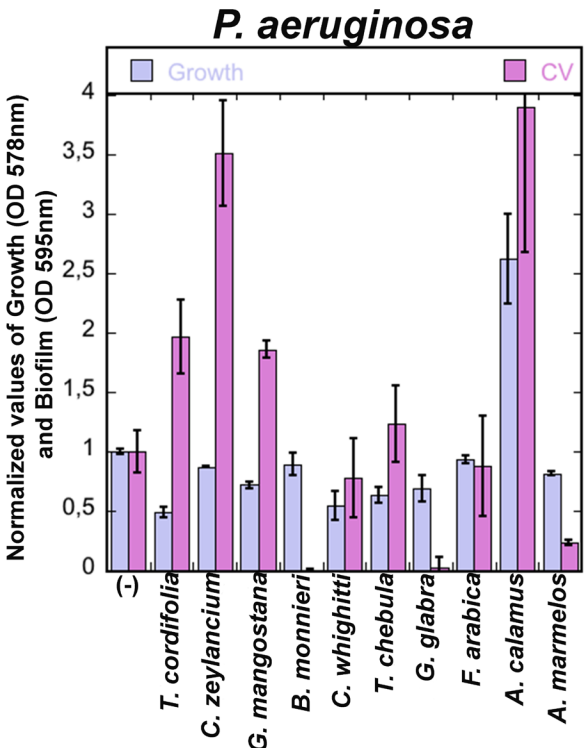


Figure 15: Anti-biofilm effect of plant extracts on submerged biofilm of pathogenic Gram negative *P. aeruginosa*. *B. monnieri* and *A. marmelos* significantly inhibited biofilm formation without showing any effect on the growth of *P. aeruoginosa*. Respective strain was grown on LB medium in 96 well plate both with and without plant extracts and incubated at 37 deg. C for 24 hrs without shaking. Unincoulated LB medium and plant extracts in LB medium without bacterial inoculation were used as control. Bacterial biomass and absorbance of crystal violet were calculated at 578 nm and 595 nm respectively. Normalized values were plotted with respect to respected conditions All the data sets are mean ± standard deviation of 3 technical replicates; experiment was repeated 3 times. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

4.7.1 *csg*BA (curli component) and

Figure. 17 shows the effects of plant extracts on *csgB* gene. Using csgB::lacZ fusion strain, direct effects of plant extracts on curli subunit synthesis were tested. Out of 10, six plant extracts, *G. mangostana, B. monnieri, C. whighitti, G. glabra, A. calamus,* and *A. marmelos* down-regulated the expression of *csgB* significantly. Interestingly, *B. monnieri* reduced the expression level to highly significant levels. These data sets were means ± standard deviations of β-galactosidase activities of 3 biological replicates.

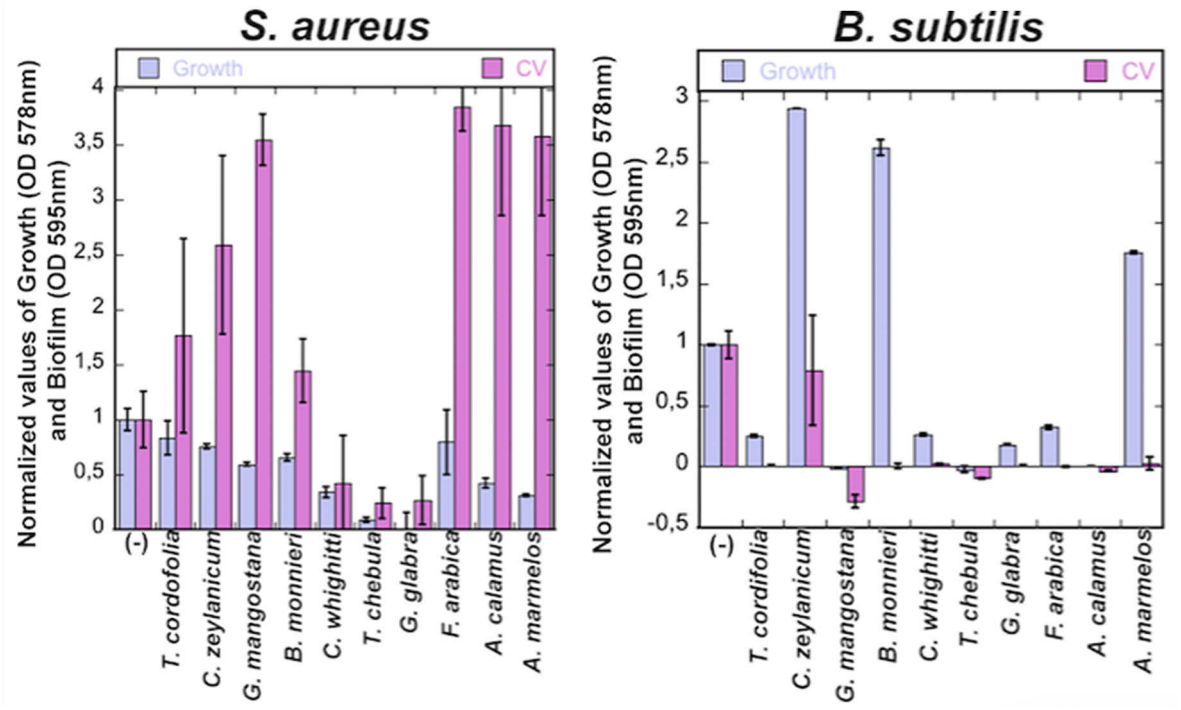


Figure 16: Anti-biofilm effect of plant extracts on submerged biofilm of Gram positive bacteria (*S. aureus* and *S. subtilis*). Both *B. monnieri* and *A. marmelos* promoted growth and completely inhibited biofilm formation of *B. subtilis*. *B. subtilis* was grown on LB medium in 96 well plate and *S. aureus* was grown on TSB with 100 mM $MgCl_2$ in 96 well plate coated with human fibronectin. Grown both in the presence and absence of plant extracts and incubated at 37 deg. C for 24 hrs without shaking. "Unincoulated medium" and "plant extracts in medium without bacterial inoculation" were used as control. Bacterial biomass and absorbance of crystal violet were calculated at 578 nm and 595 nm respectively. Normalized values were plotted with respect to control. All the data sets are mean ± standard deviation of 3 technical replicates. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

4.7.2 *dgc*C (regulating the synthesis of pEtN-cellulose)

Figure 17 also shows the effect of plant extracts on the expression level of *dgcC*. Six plant extracts, *T. cordifolia, B. monnieri, G. glabra, F. arabica, A. calamus and A. marmelos* down-regulated the expression level to an extent. Out of these, 3 plant extracts, *B. monnieri, G. glabra,* and *A. marmelos* showed the most significant downfall.

B. monnieri, G. glabra and *A. marmelos* remarkably down-regulated the expression level of both *csgB* and *dgcC*. So, based on these results, it could be concluded that only these three were selected for further studying molecular mechanisms targeting biofilm-related gene expression in *E. coli*. Rest all others were subjected to analyze the amyloidogenesis of CsgA directly. Apart from studying their effects on biofilm-related genes, these three plant extracts were also analyzed for their effects on the assembly of curli subunits (amyloidogenesis of CsgA) along

with all other plant extracts. A schematic diagram of the work plan was also shown in Figure 4.

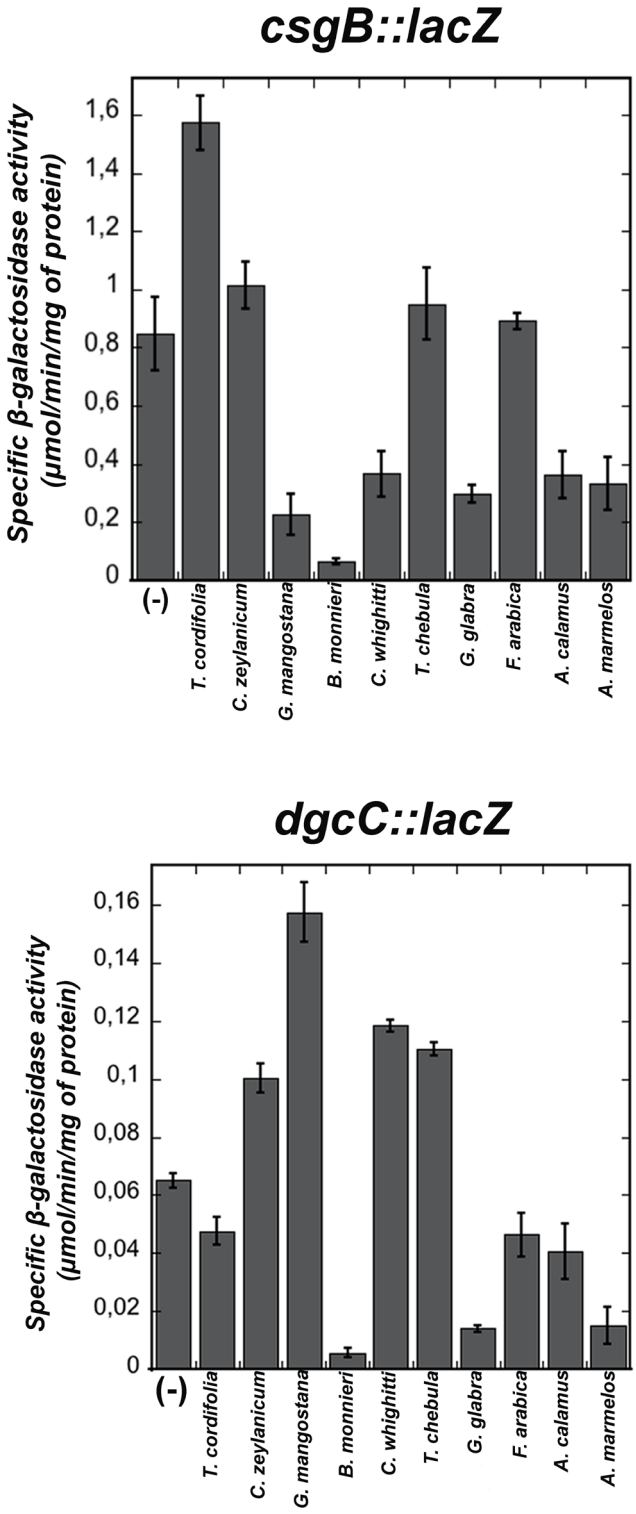


Figure 17: Effect of plant extracts on biofilm synthesis related genes *csgB* and *dgcC*. Single copy *lacZ* reporter fusion strain of *csgB* and *dgcC* was inoculated with and without plant extracts in LB medium without salt at 28 deg. C in water bath at 200 rpm for 24 hrs. Effects were analyzed on the basis of specific beta galactosidase activity (µmol/min/mg of protein)

with respect to reference. All the data sets were mean ± standard deviation of beta galactosidase activity of 3 biological replicates. *B. monnieri*, *G. glabra* and *A. marmelos* significantly reduced the expression level of both *csgB* and *dgcC*; thus, further selected for analysis of expression level of biofilm related gene expression. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

4.8 Molecular mechanisms targeted by plant extracts that affect biofilm-related gene expression in *E. coli*

As per results obtained from the previous section, only *B. monnieri, G. glabra* and *A. marmelos* down-regulated both genes directly involved in the synthesis of curli subunits (*csgB*) and pEtN-cellulose synthesis via *dgcC* in *E. coli* (Fig. 17). Based on this, it was further investigated that at which level of molecular cascade these unknown compound(s) of plant extracts were targeting to inhibit the biofilm formation. The range of targets (well explained in the schematic diagram of the work plan in Figure 4) were considered that were directly or indirectly regulating the genes involved in the synthesis of biofilm formation. As mentioned earlier, W3110 derivatives carrying single-copy *lacZ* fusions along with the target gene were used to study the expression level of targeted genes. The immunoblotting technique was also incorporated wherever needed to study the cellular level of the proteins.

4.8.1 *Bacopa monnieri, Glycyrrhiza glabra,* and *Aegle marmelos* do not affect the cellular level and activity of RpoS

Figure 18 (A) shows cellular levels of RpoS determined using Western Blotting. $RpoS^+$ (W3110) strain grown both in the presence and absence of plant extracts for 24 hrs in the LB medium without salt and samples collected showed that none of the plant extracts affected the RpoS cellular level with respect to both positive control $RpoS^+$ (without treatment with plant extracts) and negative control $RpoS^-$ (without plant extract). Figure 18 (B) shows the expression level of RpoS using *syn*P8::lacZ strain (*syn*P8 is an artificial promoter under the regulation of RpoS). There was no effect at the level of the expression level of RpoS. This clearly shows that none of the plant extracts was affecting biofilm formation via the master regulator of the stress response, RpoS.

4.8.2 *Bacopa monnieri, Glycyrrhiza glabra,* and *Aegle marmelos* downregulate the cellular level of CsgD

Figure 19 is the immunoblot analysis of the cellular level of CsgD using three selected plant extracts. The experimental setup was exactly the same as per explained in the previous section including positive and negative control. Samples collected at different time points (8^{th} hour,

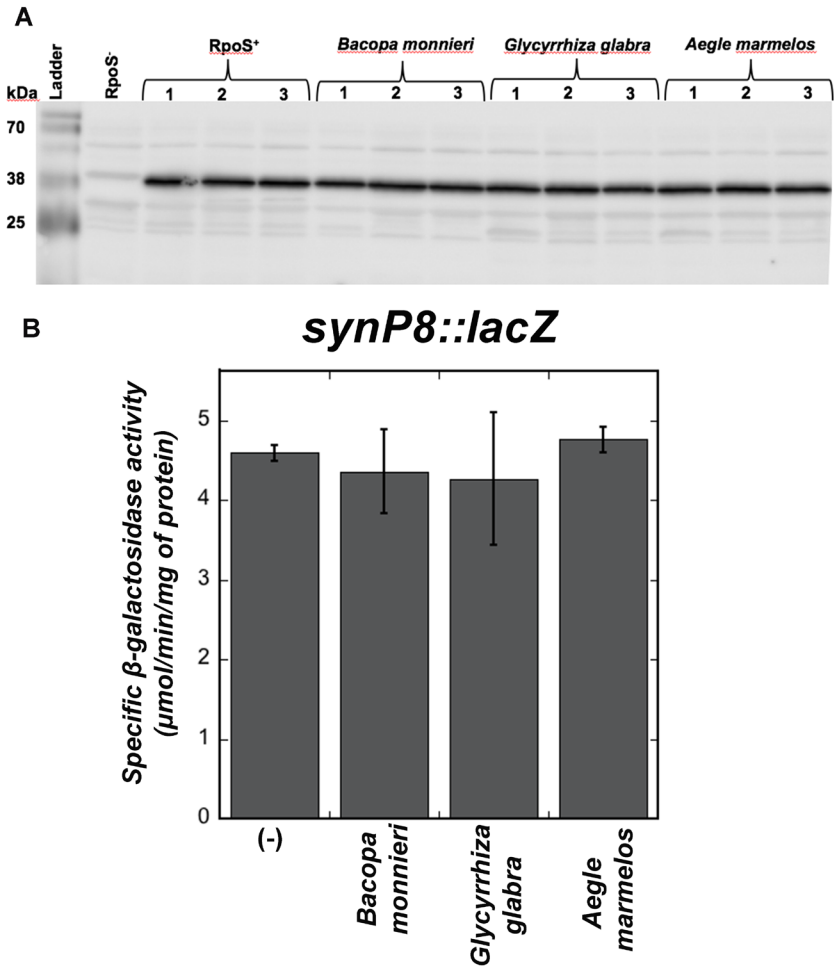


Figure 18: Effect of *B. monnieri*, *G. glabra* and *A. marmelos* on RpoS (master stress response regulator) at both cellular level and expression of the gene using immunoblot technique and single copy *lacZ* reporter fusion strain of *synP8::lacZ* respectively. None showed any significant effect on RpoS. (A) $RpoS^{+}$ (W3110) strain was grown both in the presence and absence of plant extracts in LB medium without salt at 28 deg. C in water bath at 200 rpm for 24 hrs and samples collected were analyzed for the cellular level of RpoS using immunoblotting. $RpoS^{-}$ (W3110) strain was used as negative control and 1, 2 and 3 represents the biological replicates. (B) *synP8::lacZ* was inoculated with and without plant extracts in LB medium without salt at 28 deg. C in water bath at 200 rpm for 24 hrs. Effects were analyzed on the basis of specific beta galactosidase activity (µmol/min/mg of protein) with respect to reference. All the data sets are mean ± standard deviation of beta galactosidase activity of 3 biological replicates. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

10^{th} hour, 12^{th} hour and overnight) showed that *B. monnieri* strongly affected (down-regulated or maybe completely switching off) cellular level of CsgD. Similar results were observed in *G. glabra* where CsgD level gets greatly reduced. Although the effects in the case of *Agele marmelos* were not as strong as *B. monnieri* and *G. glabra* yet it down-regulated CsgD as the thickness of the band was not as pronounced as it is there in positive control.

So, it could be concluded that the unknown compound(s) of plant extracts could be targeting at the level of CsgD. The downregulation of *csgB* and *dgcC* could be because of the downregulation of CsgD which was further under the regulation of multiple transcriptional factors (Ogasawara, Yamada, Kori, Yamamoto, & Ishihama, 2010). To untangle the mystery further, a couple of such factors were considered as follows.

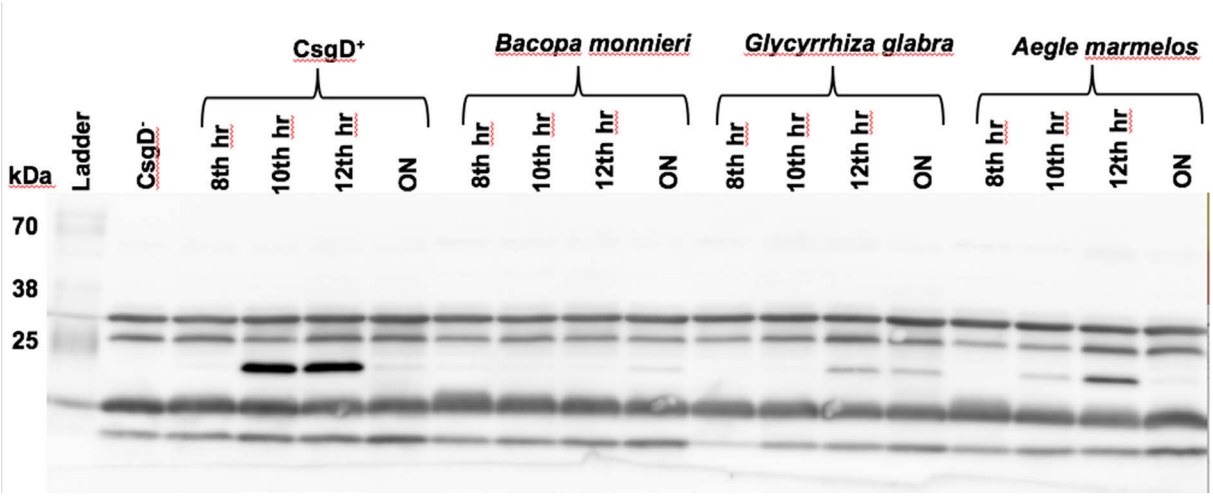


Figure 19: Effect of *B. monnieri*, *G. glabra* and *A. marmelos* on CsgD (master biofilm regulator) at cellular level using immunoblot technique. *B. monnieri, G. glabra* and *A. marmelos* strongly affected cellular level of CsgD. $CsgD^+$ (W3110) strain was grown both in the presence and absence of plant extracts in LB medium without salt at 28 deg. C in water bath at 200 rpm for 24 hrs and samples collected were analyzed for the cellular level of CsgD using immunoblotting. $CsgD^-$ strain was used as negative control and 8^{th}, 10^{th}, 12^{th} and ON (overnight) represents different sampling time points. Experiment was repeated 3 times. (NOTE: *lacZ* fusion reporter was not considered in case of *csgD* because it has highly complex regulation system and is under the control of multiple transcriptional regulators). Ref. Table 4 for optimized concentrations of plant extracts used in this experiments. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

4.8.3 None of the selected plant extracts act at the level of cell envelope stresses

RpoE is the major sigma factor involved in cell envelope stresses (Rouvière et al., 1995). DegP is a multifunctional protease which works under the control of RpoE regulon in *E. coli* (C. H. Jones et al., 2002; Lipinska, Zylicz, & Georgopoulos, 1990). Cpx is another cell envelop stress response regulatory system that also controls conversion of DegP periplasmic protease (Danese & Silhavy, 1998). *degP::lacZ* (with the background strain W3110) fusion strain was used to identify the collective response of RpoE and Cpx on cell envelop stress response by the plant extracts. For analyzing individual effects of plant extracts on RpoE and/or Cpx, one additional *degP::lacZ* strain with *cpxR* mutant was used.

Using *degP::lacZ* (background W3110), none of the selected plant extracts affected biofilm inhibition via cell envelope stress response (RpoE and Cpx) (Figure 20). As there was no effect on *degP::lacZ* (W3110) so there was no point of experimenting with *degP::lacZ* strain with *cpxR* mutation.

In addition to RpoE and Cpx, there was another envelope stress response regulator, the Rcs system, that responds to changes in charge on lipopolysaccharide or fluidity, modification in peptidoglycan biosynthesis (Farris, Sanowar, Bader, Pfuetzner, & Miller, 2010; Mitchell & Silhavy, 2019). The biofilm-dependent modulation gene, *bdm* is positively regulated by RcsB response regulator (Francez-Charlot, Castanié-Cornet, Gutierrez, & Cam, 2005). *bdm::lacZ* fusion strain was used as a reference strain to analyze the effect of plant extracts on biofilms via Rcs envelope stress response. Figure 20 showed that none of the plant extracts had any effects on CsgD via Rcs regulatory system.

So, it could be concluded that none of the stress response regulatory system was involved to affect the CsgD expression.

4.8.4 None of the selected plant extracts induces a heat shock response

Figure 21 shows that using *dnaK::lacZ* fusion reporter strain as a reference strain, no heat shock response was detected by any 3 selected plant extracts.

4.8.5 None of the selected plant extracts act by affecting c-di-GMP signaling in CsgD control

Another way by which CsgD expression level can be effected is by c-di-GMP signaling. It is a tight switch between the flagellar planktonic and biofilm phase. Using complex networking between c-di-GMP, PdeR, DgcM, and MlrA, CsgD is being regulated to control the production of biofilm components (D. O. Serra & Hengge, 2014, 2019). W3110 *pdeR dgcM* strain was used as a reference strain to analyse the effects on c-di-GMP (if any). Macrocolonies with this strain in the presence or absence of plant extracts showed that effects were independent of PdeR and DgcM (Figure 22). This means that even in the absence of PdeR and DgcM, plants were producing similar effects confirming that none of the plant extracts were targeting PdeR and DgcM to interfere with the regulation of CsgD via MlrA.

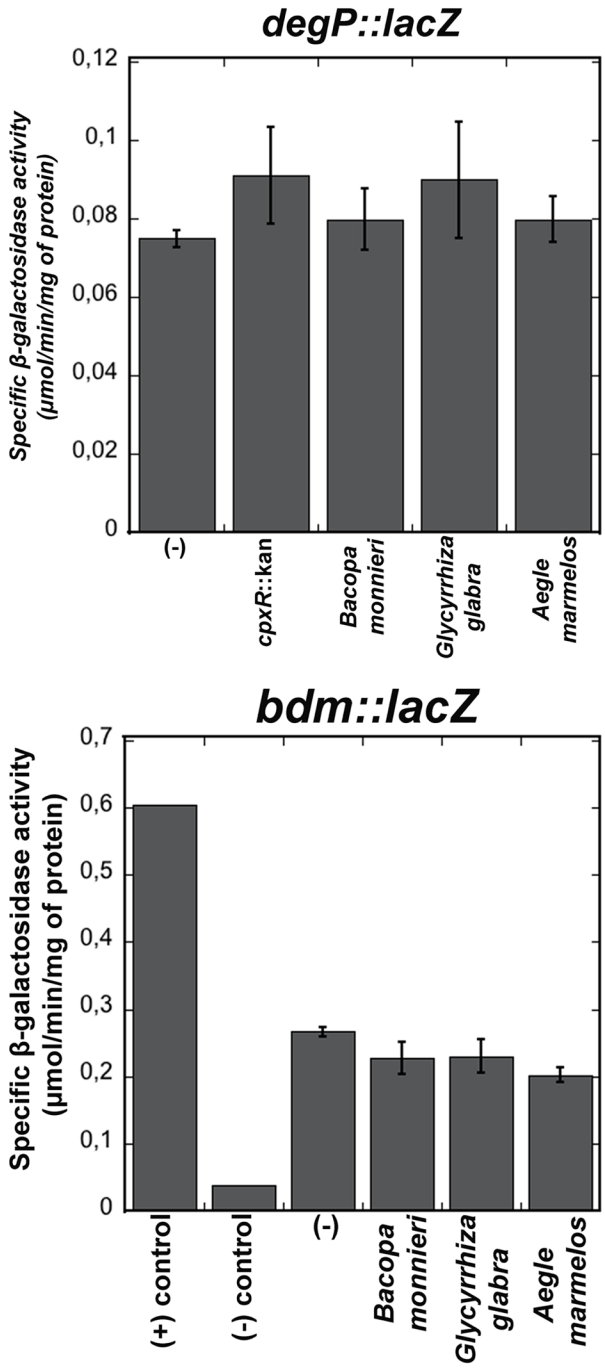


Figure 20: Effect of B. *monnieri*, *G. glabra* and *A. marmelos* on cell envelop stress response (RpoE, Cpx and Rcs). None of the stress response regulatory system was involved to affect the CsgD expression. *degP::lacZ* and *bdm::lacZ* was inoculated with and without plant extracts in LB medium without salt at 28 deg. C in water bath at 200 rpm for 24 hrs. Effects were analyzed on the basis of specific beta galactosidase activity (µmol/min/mg of protein) with respect to reference and none showed any effect at cell envelop stress response level. In case of *bdm::lacZ* reporter fusion strain, *rcsC::*cat (background strain *bdm::lacZ*) and *rcsB*::kan (background strain *bdm::lacZ*) were used as positive and negative control. All the data sets were mean ± standard deviation of beta galactosidase activity of 3 biological replicates. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

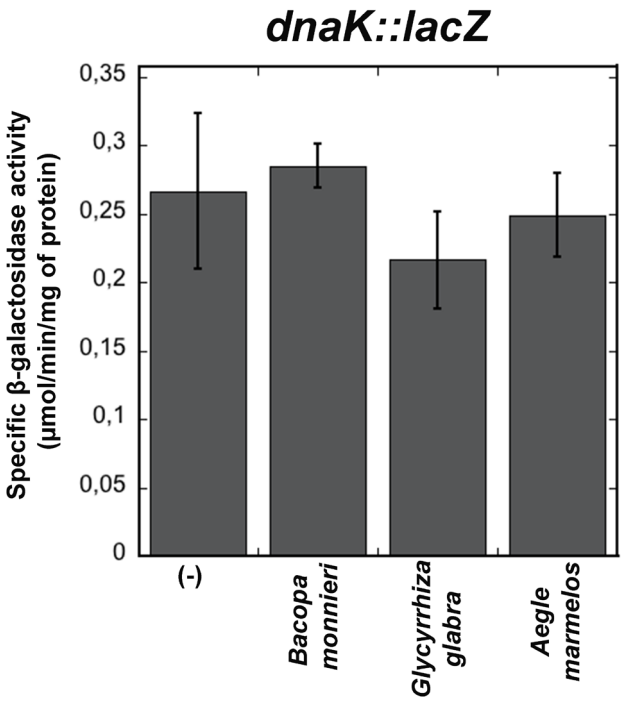


Figure 21: Effect of *B. monnieri*, *G. glabra* and *A. marmelos* on heat shock response. *dnaK::lacZ* was inoculated with and without plant extracts in LB medium without salt at 28 deg. C in water bath at 200 rpm for 24 hrs. Effects were analyzed on the basis of specific beta galactosidase activity (µmol/min/mg of protein) with respect to reference and none showed any effect at heat shock response level. All the data sets were mean ± standard deviation of beta galactosidase activity of 3 biological replicates. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

4.8.6 *Bacopa monnieri* upregulates the expression of flagellar gene cascade

The flagellar gene cascade is conversely regulated with the biofilm cascade. To assay effects on the flagellar gene cascade, level 2 and level 3 genes were tested (FliA and PdeH, respectively). Figure 23 showed that using *fliA::lacZ* and *pdeH::lacZ,* only *B. monnieri* showed remarkably high amounts of beta-galactosidase activity which means in the presence of plant extract, *B. monnieri,* flagellar gene cascade was upregulated.

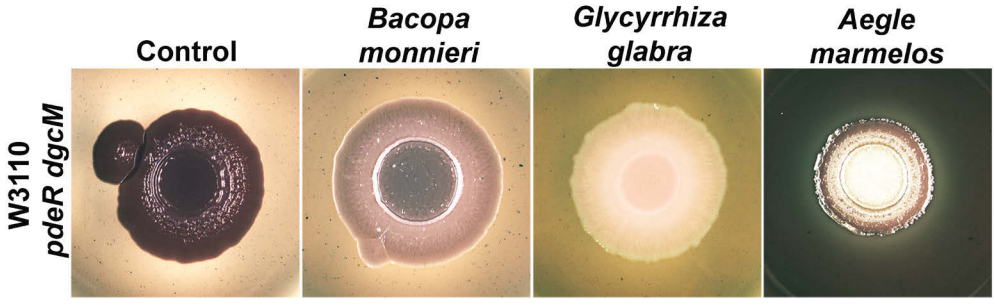


Figure 22: Effect of *B. monnieri*, *G. glabra* and *A. marmelos* on c-di-GMP using macrocolony W3110 *pdeR* and *dgcM* strain with and without plant extracts. Growth conditions and image

capturing was done as explained in figure legend 11. No effect was observed on c-di-GMP level. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

So, it is clear from the results that all the selected 3 plant extracts significantly reduced the cellular level of CsgD. This reduction neither occurred via RpoS (master stress response regulator) or envelope stress response or c-di-GMP inputs into the CsgD control nor via heat shock response. Interestingly, only one plant extract *B. monnieri* considerably upregulated the flagellar gene cascade by promoting the expression level of class 2 and class 3 genes encoding FliA and PdeH respectively which encodes flagellar sigma factor and most phosphodiesterase c-di-GMP in *E. coli*.

4.8.7 Direct effects of plant extracts on amyloidogenesis of CsgA

All plant extracts irrespective of the fact that they were altering the expression level of biofilm-related genes or not were subjected to *in vitro* amyloidogenesis of CsgA assay. To investigate if plant extracts did show some inhibition at the level of polymerization of CsgA subunits, Thioflavin T (ThT) was used which shows increased fluorescence on binding to CsgA amyloid fibers (X. Wang, D. R. Smith, J. W. Jones, & M. R. Chapman, 2007; Zhou et al., 2013). Fig. 24, 25 and 26 collectively showing the results of all the plant extracts. In this experiment EGCG which is already known for interference in amyloidogenesis of CsgA (R. Hengge, 2019) was used as a positive control. All the plant extracts inhibit the CsgA polymerization *in vitro* (there was no increase in fluorescence, whenever an extract was in the assay). All the plant extracts (except that of *C. zeylanicum*) have strong auto-fluorescence, probably they may contain flavonoids which are auto-fluorescence or any other auto-fluorescent components. In the case of *B. monnieri* and *G. mangostana*, CsgA seems to quench some of the auto-fluorescence components which make it likely that the auto-fluorescent components could be flavonoids. In some cases, the auto-fluorescence of an extract (*T. cordifolia, G. glabra, F. arabica,* and *A. marmelos*) can increase later during the assay in a manner that was somehow modulated by the presence of CsgA.

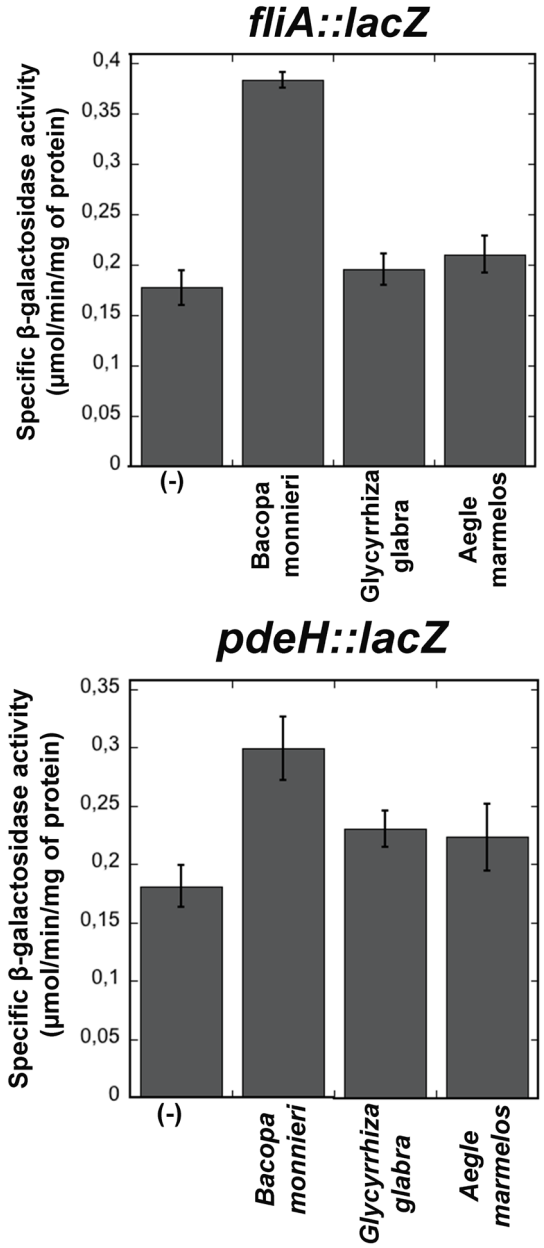


Figure 23: Only *B. monnieri* upregulated flagellar gene cascade. *fliA::lacZ* and *pdeH::lacZ* was inoculated with and without plant extracts in LB medium without salt at 28 deg. C in water bath at 200 rpm for 24 hrs. Effects were analyzed on the basis of specific beta galactosidase activity (µmol/min/mg of protein) with respect to reference and found that *B. monnieri* upregulated the *fliA* and *pdeH* expression to a large extent. All the data sets are mean ± standard deviation of beta galactosidase activity of 3 biological replicates. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

To confirm that plant extracts did not pose any hindrance in the assay by interacting with ThT and blocking its interactions with CsgA, Transmission Electron Microscopy (TEM) was performed selecting 2 plant extracts, *C. zeylanicum* and *G. mangostana,* both with CsgA and without CsgA in the presence of ThT images were captured. Figure 27 shows the control CsgA

with ThT (without any plant extract) was producing a network of amyloid fibers whereas none of such fibrous network was seen in the presence and absence of CsgA with plant extracts. Thus, it could be concluded that plant extracts were not posing any blockage in the assay by interacting with ThT and thus, making it unavailable for CsgA interaction and generating false results, but rather efficiently interfere with the formation of amyloid fibers.

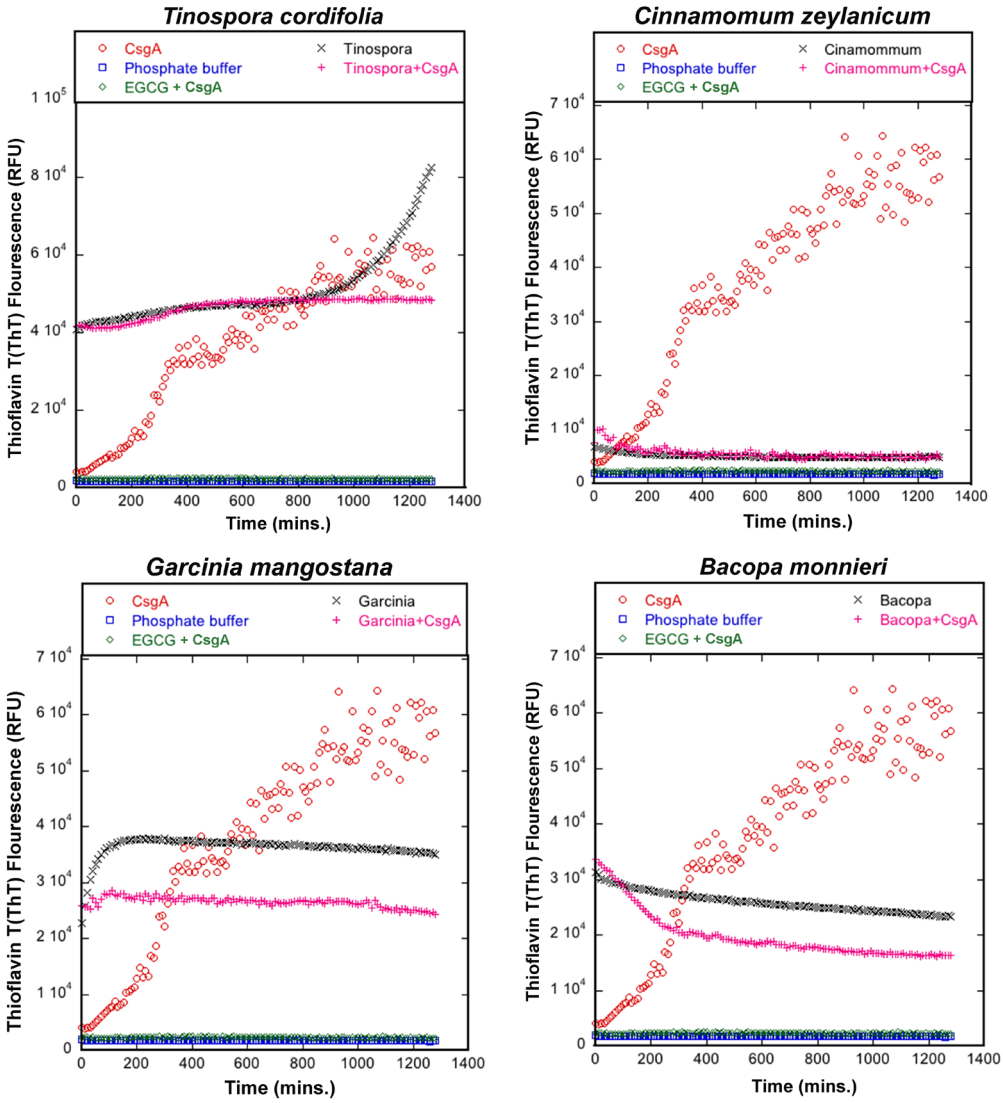


Figure 24 *In vitro* inhibition of CsgA polymerization by *T. cordifolia, C. zeylanicum, G. mangostana,* and *B. monnieri*. Reaction mixture was prepared using 20 µM of Thioflavin T (ThT) with 100 µL of freshly prepared CsgA in the presence and absence of plant extracts in a non-treated, flat-bottom, non-binding, opaque Greiner 96- well microtiter plate. After sealing with transparent sticker, it was read at excitation wavelength to be 438 nm, the emission wavelength 495 nm and a cut-off filter at 475 nm. The program was set to read the plate every 10 mins at room temperature over the course of 24 hours. Potassium phosphate buffer and untreated purified CsgA were used as controls. Epigallocatachin gallate (EGCG) of 50 µg/mL with CsgA was used as positive control. Additional control for each plant extract containing phosphate buffer along with optimized concentration of extracts (without CsgA) was also considered. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

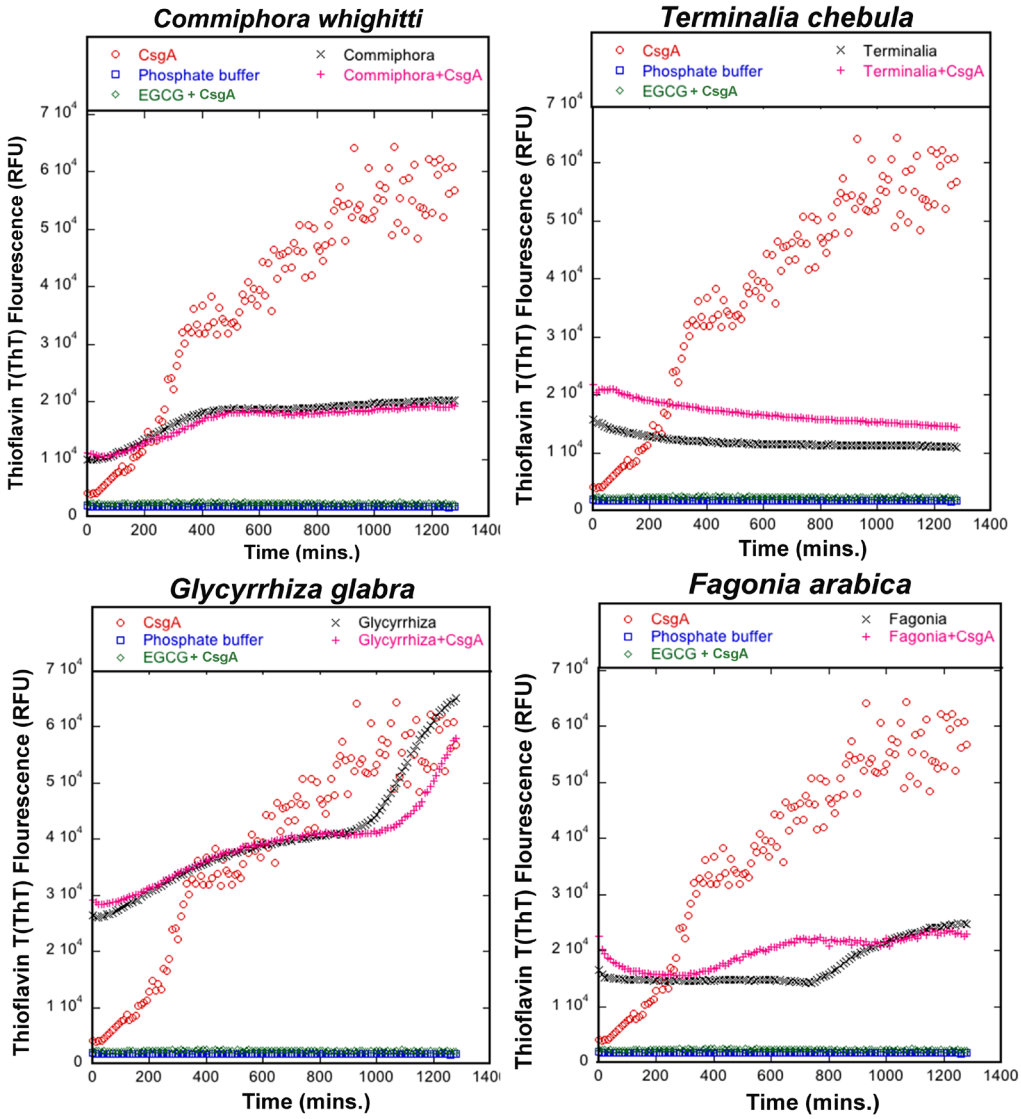


Figure 25*: In vitro* inhibition of CsgA polymerization by *C. whighitti, T. chebula, G. glabra,* and *F. arabica.* Reaction set up is same as explained in figure legend 24. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

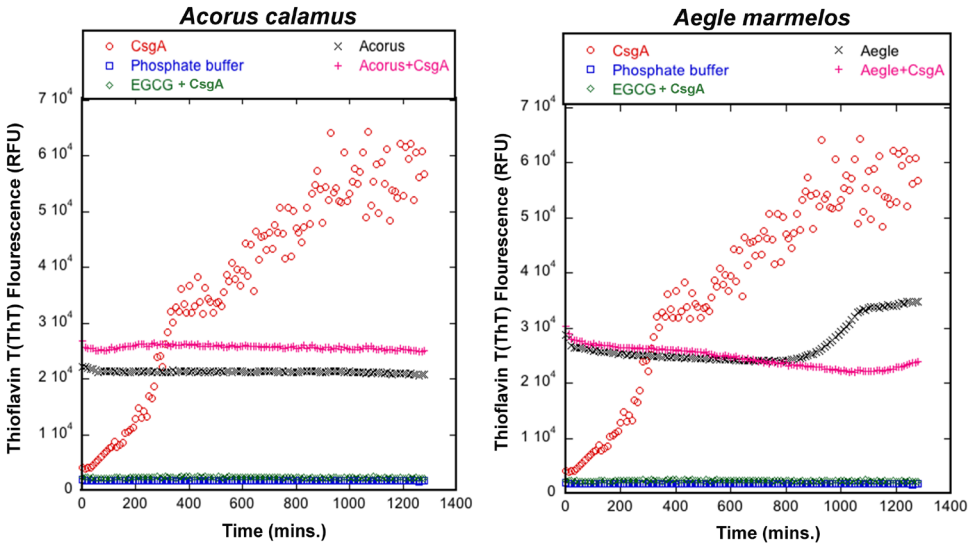
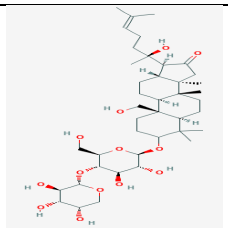
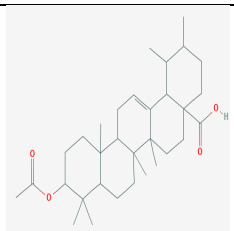


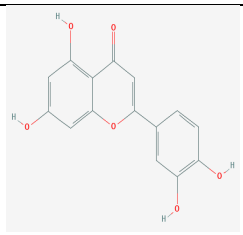
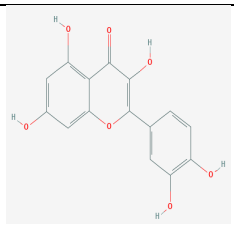
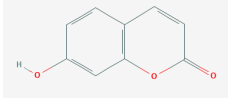
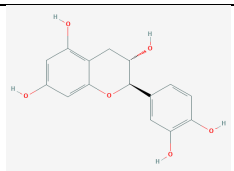
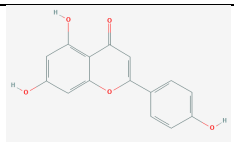
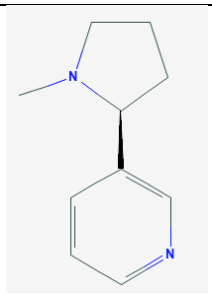
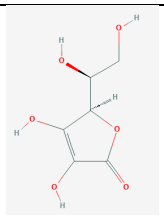
Figure 26: *In vitro* inhibition of CsgA polymerization by *A. calamus* and *A. marmelos.* Reaction set up is same as explained in figure legend 24. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

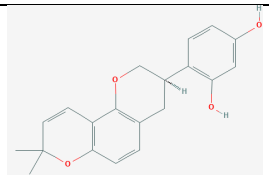
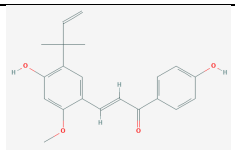
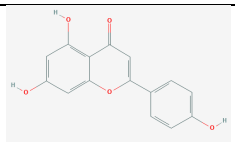
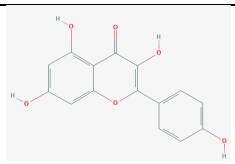
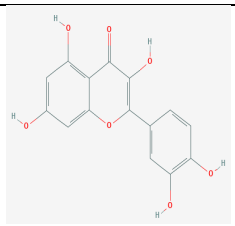
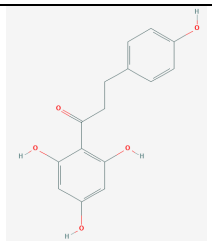
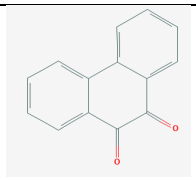
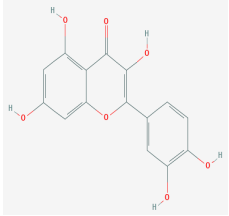
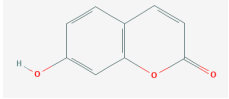
4.9 Pure compounds as potential anti-biofilm compounds

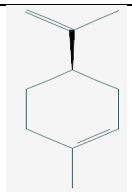
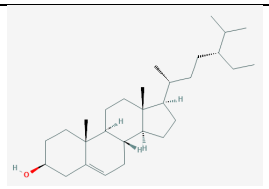
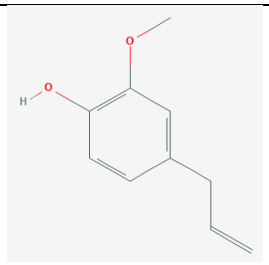
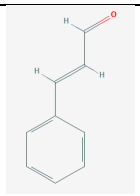
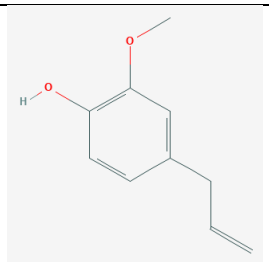
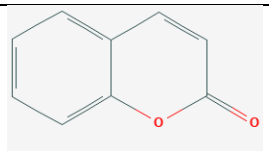
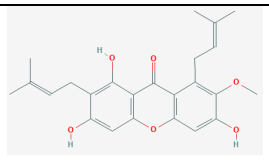
Table 7 contains list of compounds present in the plant extracts used in this project already known to have anti-biofilm effects against various bacterial species and other biological activities. On the basis of this literature, two major compounds were selected, Bacoside A (present in *B. monnieri*) and Glabridin (present in *G. glabra*) to test for their anti-biofilm effects on *E. coli* K-12.

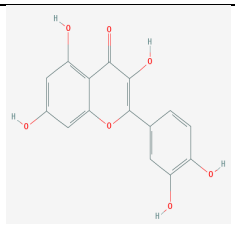
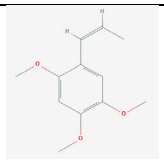
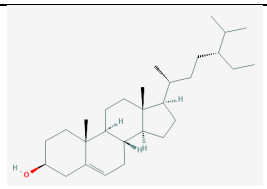
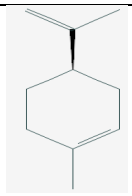
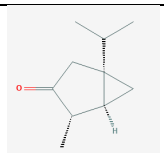
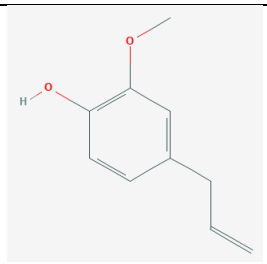
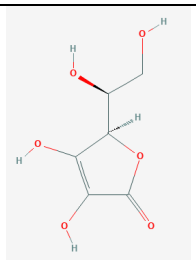
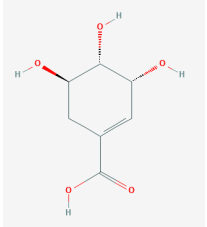
Table 7 List of compounds extracted from the plants (used in this study) known to have anti-biofilm and neuroprotective activities.

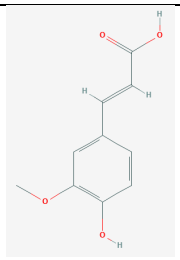
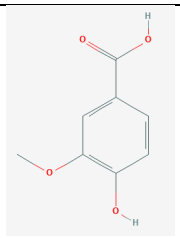
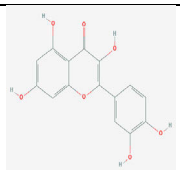
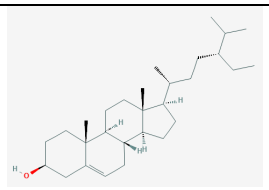
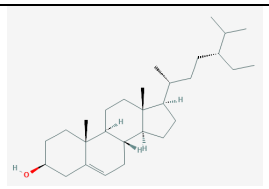
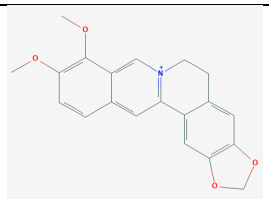
Name of plants	Name of compounds	Biological activity	Structure	References
Bacopa monnieri	**Bacoside A (major constituent)**	Anti-biofilm (against *S. aureus* and *P. aeruginosa*) and neuroprotective		(Abdul Manap et al., 2019; Parai, Islam, Mitra, & Mukherjee, 2017)
	Ursolic acid	Anti-biofilm (against *E. coli* K-12), inducing genes for chemotaxis and mobility (*motAB)* and also neuroprotective		(Habtemariam, 2019; Jeyasri, Muthuramalingam, Suba, Ramesh, & Chen, 2020; Dacheng Ren et al., 2005)

	Luteolin	Anti-biofilm (against *E. coli* K-12) and neuroprotective		(Kamkaew, Paracha, Ingkaninan, Waranuch, & Chootip, 2019; Pruteanu et al., 2020; Rajasekaran, 2014; Xu et al., 2014)
	Quercetin	Anti-biofilm (against *E. coli* K-12) and neuroprotective		(Bhatia, Dhuna, Dhuna, Kaur, & Singh, 2017; Costa, Garrick, Roquè, & Pellacani, 2016; Kapil et al., 2014; Pruteanu et al., 2020)
	Umbelliferone	Anti-biofilm (against methicillin-resistant *Staphylococcus epidermidis*) and neuroprotective		(Bhatia et al., 2017; KARUTHA PANDIAN et al., 2019; Subramaniam & Ellis, 2013)
	Catechin	Anti-biofilm (against *Eikenella corrodens*) and neuroprotective		(Bhatia et al., 2017; Mandel & Youdim, 2004; Matsunaga et al., 2010)
	Apigenin (major constituent)	Anti-biofilm (against *Streptococcus mutans*) and neuroprotective		(Aguiar & Borowski, 2013; Koo et al., 2003; Nabavi et al., 2018; Rajasekaran, 2014)
	Nicotine (one of the major alkaloids)	**Increases cell attachment and biofilm formation of *S. aureus* and *S. epidermidis***		(Jeyasri et al., 2020; Shi et al., 2019; Srivastava, Srivastava, Pandey, Khanna, & Pant, 2019; Wu et al., 2018)
	Ascorbic Acid	inhibit bacterial quorum sensing and other regulatory mechanisms underpinning biofilm development		(Jeyasri et al., 2020; Pandit et al., 2017)

Glycyrrhiza glabra	**Glabridin (major constituent)**	**Anti-biofilm (against *E.coli* K-12)**		**This project**
	licochalcone A	Anti-biofilm (against *Candida albicans*) and neuroprotective		(Fu et al., 2004; Liu, Ma, Wei, & Fan, 2018; Messier & Grenier, 2011)
	Apigenin	Anti-biofilm (against *Streptococcus mutans*) and neuroprotective		(Koo et al., 2003; Nabavi et al., 2018; B. Singh, Mungara, Anandjiwala, & Nivsarkar, 2009)
	Kaempferol	Anti-biofilm (against *S. aureus*) and neuroprotective		(S. Li & Pu, 2011; Ming et al., 2017; B. Singh et al., 2009)
	Quercetin	Anti-biofilm (against *E. coli* K-12) and neuroprotective		(Costa et al., 2016; Pruteanu et al., 2020; B. Singh et al., 2009)
	Phloretin	Anti-biofilm (against *Escherichia coli* O157: H7) and neuroprotective		(Barreca et al., 2017; Park, Jeong, Lee, & Lee, 2012; Sahoo, Gouda, Das, Pandey, & Bhattacharyay, 2020)
Aegle marmelos	Phenanthrenequinone (novel quinone)	Anti-biofilm (against *E. coli*, *S. typhii* and *P. aeruginosa)*		(Rejiniemon et al., 2014)
	Quercetin	Anti-biofilm (against *E. coli* K-12) and neuroprotective		(Costa et al., 2016; Pruteanu et al., 2020; Siddiqui, Mujeeb, Amir, & Husain, 2012)
	Umbelliferone	Anti-biofilm (against methicillin-resistant *Staphylococcus*		(KARUTHA PANDIAN et al., 2019; Siddiqui et al., 2012;

		epidermidis) and neuroprotective		Subramaniam & Ellis, 2013)
	D-Limonene	Inhibiting *E. coli* biofilm formation through the suppression of curli		(A. R. Patel, Garach, Chakraborty, & Kamath, 2012)
	β-Sitosterol	Inhibiting biofilm and motility of *Escherichia coli* O157:H7 (EHEC)		(A. R. Patel et al., 2012; Amit Vikram, Jayaprakasha, Uckoo, & Patil, 2013)
	Eugenol	Anti-biofilm (against methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* clinical strain) and neuroprotective		(Maity, Hansda, Bandyopadhyay, & Mishra, 2009; Wie et al., 1997; Yadav, Chae, Im, Chung, & Song, 2015)
Cinnamomum zeylanicum	Cinnamaldehyde	Anti-biofilm (against *P. aeruginosa)* and neuroprotective		(Rao & Gan, 2014; Topa et al., 2018; J. Zhao et al., 2015)
	Eugenol	Anti-biofilm (against methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* clinical strain) and neuroprotective		(Rao & Gan, 2014; Wie et al., 1997; Yadav et al., 2015)
	Coumarin	Anti-biofilm (against *E. coli*, *Staphylococcus aureus* and *P. aeruginosa*) and neuroprotective		(Rao & Gan, 2014; Reen, Gutiérrez-Barranquero, Parages, & O´Gara, 2018; C. Wang et al., 2012)
Garcinia mangoatana	**α**-mangostin	Anti-biofilm (against *Staphylococcus aureus*)		(Phuong et al., 2017)

Commiphora whighitti	Quercetin	Anti-biofilm (against *E. coli* K-12) and neuroprotective		(Costa et al., 2016; Pruteanu et al., 2020; Sarup, Bala, & Kamboj, 2015)
Acorus calamus	β -asarone	Antifungal and sub-inhibitory concentrations are Anti-biofilm against *Candida albicans*		(Chandra & Prasad, 2017; Rajput & Karuppayil, 2013)
	β-Sitosterol	Inhibiting biofilm and motility of *Escherichia coli* O157:H7 (EHEC)		(Chandra & Prasad, 2017; Amit Vikram et al., 2013)
	D-Limonene	Inhibiting *E. coli* biofilm formation through the suppression of curli		(Chandra & Prasad, 2017; R. Wang, Vega, Xu, Chen, & Irudayaraj, 2018)
	α-Thujone	Anti-biofilm		(Chandra & Prasad, 2017; Jaafar, Mitri, & Na'was, 2018)
Terminalia chebula	Eugenol	Anti-biofilm (against methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* clinical strain) and neuroprotective		(Riaz M, 2017; Yadav et al., 2015)
	Ascorbic acid	inhibit bacterial quorum sensing and other regulatory mechanisms underpinning biofilm development		(Pandit et al., 2017; Riaz M, 2017)
	Shikimic acid	Anti-biofilm against *S. aureus*		(Bai, Zhong, Wu, Elena, & Gao, 2019) (Riaz M, 2017)

	Ferulic acid	Anti-biofilm against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Listeria monocytogenes*		(Borges, Saavedra, & Simões, 2012; Riaz M, 2017)
	Vanillic acid	Anti-biofilm against carbapenem-resistant *Enterobacter hormaechei*		(Qian et al., 2020; Riaz M, 2017)
	Quercetin	Anti-biofilm (against *E. coli* K-12) and neuroprotective		(Costa et al., 2016; Pruteanu et al., 2020; Riaz M, 2017)
	β-Sitosterol	Inhibiting biofilm and motility of *Escherichia coli* O157:H7 (EHEC)		(Riaz M, 2017; Amit Vikram et al., 2013)
Tinospora cordifolia	β-Sitosterol	Inhibiting biofilm and motility of *Escherichia coli* O157:H7 (EHEC)		(Saha & Ghosh, 2012; Amit Vikram et al., 2013)
	Berberine	Anti-biofilm against *S. epidermidis*		(X. Wang et al., 2009) (Saha & Ghosh, 2012)

4.9.1 Glabridin (Present in: *Glycyrrhiza glabra*)

Glabridin, the major flavonoid of *G. glabra* have neuroprotective effects both *in vivo* and *in vitro* (Yu et al., 2008). Apart from that, it is also known for its many other biological activities. Its major reported biological properties include anti-inflammatory properties, prevention of low-density lipoprotein (major cholesterol carrier in human serum) oxidation, shows assay-dependent estrogenic properties (potential phytoestrogen), can modulate bone disorders (Simmler, Pauli, & Chen, 2013). As plant flavonoids are also known for their anti-biofilm

activity (Lopes, Dos Santos Rodrigues, Magnani, de Souza, & de Siqueira-Júnior, 2017; Raorane et al., 2019; A. Vikram, Jayaprakasha, Jesudhasan, Pillai, & Patil, 2010) against various microbes, and it also has neuroprotective effects so, based on our rationales for the selection of these plants flavanoid, Glabridin was selected to test the biofilm activity against AR3110.

Figure 28 (A) is the chemical structure of Glabridin and Figure 28 (B) showing the dose-effect of Glabridin on biofilm formation of AR3110. Strikingly, the color change of the macrocolony from dark red to pink in the presence of Glabridin depicts that it has some effect on curli fiber synthesis. Typical colony morphology with ridges was distorted. As the results are interesting so it is sensible to study its effect in more detail using the schematic work plan which is followed for all the plant extracts. As the pure compound was dissolved in DMSO, so, only DMSO was also used as control.

4.9.2 Bacoside A (present in: *Bacopa monnieri*)

Traditional Indian medicinal plant *B. monnieri* has mixed saponins known as Bacoside A which has been used to treat various nervous disorders and also used to promote memory (Bammidi, Volluri, Chippada, Avanigadda, & Vangalapati, 2011; Limpeanchob, Jaipan, Rattanakaruna, Phrompittayarat, & Ingkaninan, 2008). It has therapeutic effects in controlling the diseases associated with amyloid proteins such as Alzheimer's disease (Apetz, Munch, Govindaraghavan, & Gyengesi, 2014; Holcomb et al., 2006).

Figure 29 (A, B, C and D) are showing results obtained with Bacoside A. Figure 32 (A) shows that bacoside A did not have a strong effect either on curli or pEtN-cellulose. The colony size of AR3110 was large in the presence of Bacoside A than control and a minor effect was seen on curli fibers. As the colony was spreading so it was decided to test it further if posing any alternation in the flagellar gene cascade (Figure 29 B). It did not have any effect on *fliA* and *pdeH*. Figure 29 (C) shows that Bacoside A enhanced the *in vitro* polymerization of CsgA. Although it did not mean that this plays a role *in vivo* too, because there CsgA is efficiently templated into the amyloid structure by CsgB. Figure 29 (D) is the chemical structure of Bacoside A.

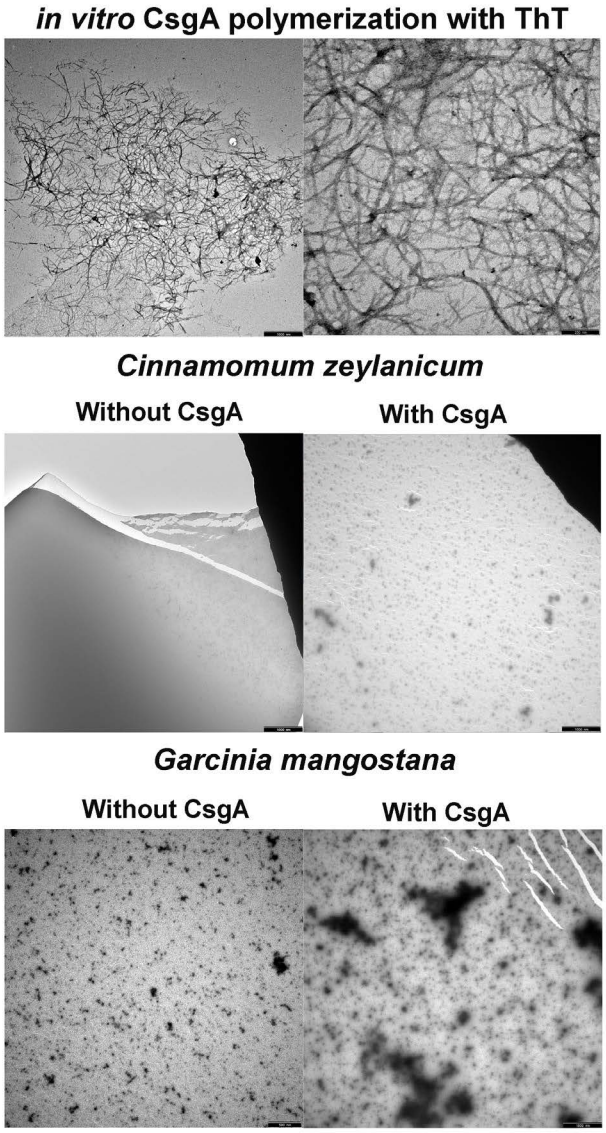


Figure 27: Plant extracts are not blocking the interactions between CsgA and Thiflavin T (ThT) as per observed under Transmission Electron Microscope (TEM). Negative staining TEM was performed on curli samples. (A) Reaction mixture of CsgA with ThT after incubation. (B) reaction mixture of *G. mangostana*, ThT with and without CsgA (C) reaction mixture of *C. zeylanicum*, ThT with and without CsgA were applied on Formvar film coated copper grids having 300-mesh (Electron Microscopy Sciences) for 2 mins. Deionized with water and negatively stained for 90 sec with 2% uranyl acetate. After air drying microscopy was performed. No visuals of fiber network of CsgA were detected with plant extracts as what is seen in CsgA control. Ref. Table 4 for optimized concentrations of plant extracts used in this experiments.

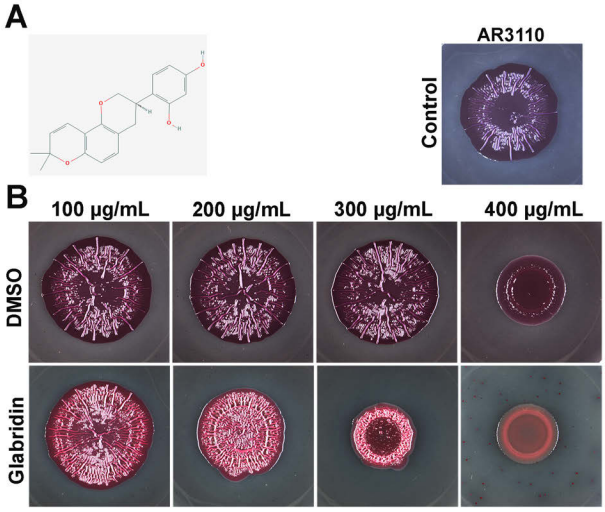


Figure 28: Effects of Glabridin on macrocolonies of AR3110. (A) Structure of Glabridin (B) Effect on macrocolonies. It had strong effect on AR3110 but not as strong as *G. glabra*, source plant.

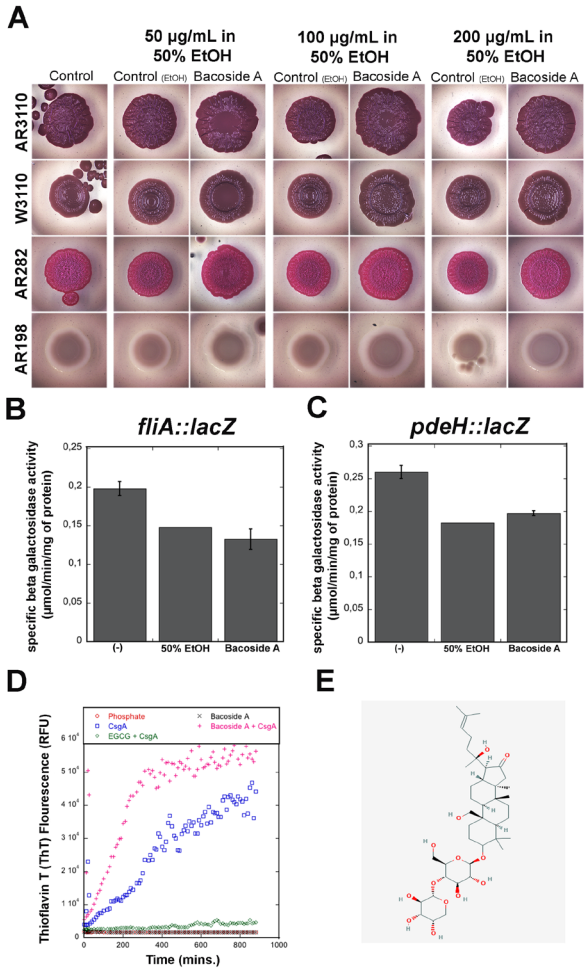


Figure 29: (A) Effects on macroconies of E. coli. It did not show effective results on macroconies. (B) & (C) No effects on flagellar gene cascade. (D) Promoted CsgA in vitro polymerization; (E) structure of Bacoside A.

5 Discussion

As per the Bulletin of the World Health Organization (WHO), 2008, 80% of the African population rely on traditional herbal medicines to alleviate and treat diseases (Tilburt & Kaptchuk, 2008). The popularity of these traditional medicinal market is increasing and approaching US$ 60 billion (Tilburt & Kaptchuk, 2008). Both developing and developed countries such as China, India, Nigeria, the United States of America (USA), and also World Health Organization (WHO) investing a lot in the research on traditional medicines (Tilburt & Kaptchuk, 2008). Plant products are being explored for their antimicrobial agents from a long time ago (Cowan, 1999).

In this study, 10 ayurvedic plants from Indian origin were explored for their anti-biofilm activity both on agar plates and submerged biofilms on 96 well plates against Gram-positive (*Bacillus subtilis*) and Gram-negative (*E. coli* K-12) bacteria as model organisms. To evaluate the broad-spectrum range of anti-biofilm activity, a set of pathogenic bacterial strains (both Gram-positive and Gram-negative) were also tested. In addition to this, molecular targets of plant extracts are also unraveled using a set of molecular genetic and various biochemical assays with *E. coli* K-12 as a model bacterial strain.

5.1 Effects of plant extracts on growth, cell division and biofilm formation of commensal and pathogenic *E. coli*

5.1.1 Is *E. coli* entering the stationary growth phase in the presence of some plant extracts?

With some of the plant extracts, the growth of *E. coli* K-12 (Fig. 5, 6 and 7) seems to get stuck in post exponential growth phase and seems like never entering the stationary phase properly in a liquid culture which was further verified with their cfu/mL after 24 hrs of incubation (Fig. 8). Surprisingly, cfu/mL is strongly elevated with almost all the plant extracts, in comparison to untreated *E. coli* culture within 24 hr of incubation. This means that cells tend to divide and re-divide and either or may never into the physiological stationary phase. With *C. zeylanicum*, *C. whighitti*, *T. chebula,* and *G. mangostana,* a long lag phase of growth is seen in a liquid culture which indicates that these plants may have some toxicity which delayed the exponential phase/ elongated lag phase of growth in *E. coli*. Similar toxicity is also observed with the plant extracts of *T. chebula* and *G. mangostana* with higher concentrations on macrocolonies on Congo red agar plates, depicting that the anti-biofilm effect is dose-dependent and at higher concentrations, these plant extracts can inhibit the bacterial growth.

5.1.2 Effect of plant extracts on biofilms of *E. coli* K-12

There are two highly conserved biochemical interactions which are involved in biofilm community establishment; production of amyloid biofilm matrices (Larsen et al., 2007) and c-di-GMP (the second messenger bis-(3′,5′)-cyclic diguanosine monophosphate) (Regine Hengge, 2009; Jenal & Malone, 2006; Jenal, Reinders, & Lori, 2017; Römling & Amikam, 2006; Whitney & Howell, 2013). All the plant extracts tested in this study affected amyloid fibrils and pEtN-cellulose on macrocolonies of commensal *E. coli* K-12, as wrinkled morphotype which depends on the high production of amyloid curli fibers and pEtN-cellulose (D. O. Serra et al., 2013) get greatly impaired on Congo red plates in the presence of plant extracts (Fig. 9 A, B, C, D, E, F, G, H, and J). Amyloid is highly diverse and abundant in many phyla such as Proteobacteria (Alpha-, Beta-, Gamma-, and Deltaproteobacteria), Bacteriodetes, Chloroflexi and Actinobacteria and could also be there in other phyla (Larsen et al., 2007), so, amyloid targeting plant extracts or pure compounds could be potentially beneficial in controlling the biofilm formation in diverse bacterial species. Results obtained in this project are supporting this rationale as all the plant extracts are affecting the amyloid fibers with or without showing any effect on pEtN-cellulose. In support to this, it has already been documented that EGCG which is showing highly promising results on macrocolonies on *E. coli* K-12 (D. O. Serra et al., 2016) also found to be effective on related amyloid fibers from other bacterial species like *Pseudomonas aeruginosa,* which is producing Fap fibers (for functional amyloids in Pseudomonas), involved in pathogenesis and cellular adhesins (Stenvang et al., 2016).

5.1.3 Diversity of biofilm composition within the same bacterial species

Attachment and biofilm formation in bacteria depends on several factors such as growth medium, substratum, and cell surfaces (Donlan, 2002). So, all the ten plant extracts were further investigated for their anti-biofilm activities on submerged biofilms formed on 96 well plates (Fig 13, 14 & 15). For initial attachment to plastic surface of dish plate, *E. coli* needs flagella and type 1 fimbriae (Pratt & Kolter, 1999). Qualification of biofilm in the presence and absence of plant extracts was done by the classical method using crystal violet staining (G. A. O'Toole, 2011). To distinguish between the effects of plant extracts on biofilm formation and on bacterial growth, overall optical density at 578 nm was also monitored for bacterial growth after incubation of 24 hr at 37 deg. C. Data obtained in this study with different species (*E. coli*, *B. subtilis*, *P. aeruginosa,* and *S. aureus*) and even different strains of the same species (*E. coli* K-12, EAEC, and UPEC) supports the diversity of composition of biofilm matrix. Most

surprisingly, *B. monnieri* strongly affected the submerged biofilms of *E. coli* K-12 strain AR3110 and pathogenic strains EAEC and UPEC. On the other hand, *G. glabra* eliminated the biofilm of *E. coli* K-12 strain AR3110 with no effect on EAEC and promoting biofilm formation in UPEC. This data is supporting that there could be diversity in their matrix composition. So, matrix diversity could be strain-specific which is also documented previously by Pruteanu with anti-biofilm studies with flavonoids (Pruteanu et al., 2020)

5.1.4 Curli fibers, the major anti-biofilm target by plant extracts

All the plant extracts showed strong effects on curli fibers with and without having any effect on cellulose but none showed any effect on pEtN-cellulose without showing any effect on curli. It could be because they also have cellulose as their major component and targeting bacterial cellulose can lead to damaging their building material. Although, bacterial cellulose is chemically modified having pEtN-cellulose (Thongsomboon et al., 2018) yet the similarity in basic chemical nature could be devastating for them and thus leads to less anti-cellulose strategies by plants to prevent the colonization of bacteria on their surfaces with evolution. *B. monnieri*, *F. arabica* and *A. calamus* have only affected curli fibers without effecting pEtN-cellulose on macrocolonies of *E. coli* (Fig. 9 D, H and J). Just as EGCG affects both curli (which is involved in adherence of bacteria to plant surfaces) and pEtN-cellulose on macrocolony Congo red agar plates (D. O. Serra et al., 2016; Yaron & Römling, 2014) many plant extracts used in this study also reduced pEtN-cellulose along with curli of *E. coli* K-12 biofilms. So, curli fibers are the major component of the bacterial *E. coli* K-12 to be targeted by the plant extracts.

5.1.5 Extracytoplamic biofilm inhibition, a convenient anti-biofilm strategy by plant extracts

As all the plant extracts strongly affected the curli fibrils on macrocolony agar plates, interestingly, they all have strong potential to interfere with the assembly of amyloid fibers *in vitro* on 96 well plate (Fig 24, 25, and 26) as per data obtained by biochemical assay of *in vitro* CsgA polymerization. It has already been found that many flavonoids and polyphenols such as EGCG are anti-amyloidogenesis and in addition to this, EGCG also has a secondary target and down-regulates CsgD expression which in turn is required for the synthesis of curli subunits; via activation of RpoE stress response (R. Hengge, 2019; Pruteanu et al., 2020; D. O. Serra et al., 2016). In the case of EGCG reported by Serra and his co-workers in 2016 and most recently flavonoids from plant origin by Pruteanu and her co-workers in 2020, it has been clarified that anti-amyloidogenesis and cell envelop stress responses seems to be more efficient anti-biofilm

targets (Pruteanu et al., 2020) because both, the hindering of amyloidogenesis and/or the signaling for RpoS activation (triggered by protein folding defects in the cell envelope of *E. coli)* are extracytoplasmic and compounds do not have to enter the cell (Pruteanu et al., 2020). So, this could be the reason that most of the plant extracts showed strong anti-amyloidogenic activity as it is easy and most convenient from the perspective of the plant. It is also noticed that all the plant extracts (except *C. zeylanicum*) alone have some fluorescence depicting that plants may have some auto-fluorescence compounds in them.

5.1.6 Effects on biofilm-related gene expression are less prominent anti-biofilm strategy by plant extracts

Only a few plants extract such as *B. monnieri*, *G. glabra,* and *A. marmelos* have clear and significant effects on biofilm-related gene expression and the reason behind this could also be the same, as to produce effects at the genetic level, compounds have to cross the barriers of cell wall and membrane which is not easy for a diverse variety of anti-biofilm chemical compounds. Aqueous ethanolic extracts of *B. monnieri*, *G. glabra,* and *A. marmelos* remarkably reduced the expression level of genes required for the synthesis of curli and pEtN-cellulose, *csgB,* and *dcgC* respectively (Fig 17). Other plant extracts are either upregulating/downregulating the expression of genes, *csgB* or *dcgC* but none out of them are collectively downregulating both *csgB* and *dcgC*. Many plant-derived pure compounds are already known to have their effects at biofilm-related genes such as ECGC in the case of *E. coli* K-12 (D. O. Serra et al., 2016), *t*-resveratrol and oxyresveratrol represses curli genes and motility genes and decreased swarming motility and curli fimbriae formation in enterohemorrhagic *E. coli* (J.-H. Lee et al., 2019), ginkgolic acid C15:1 repressed curli genes and prophage genes in EHEC (J. H. Lee, Kim, Ryu, Cho, & Lee, 2014), and prenylated flavanones propolin D reduced fimbriae production by E. coli O157:H7 and repressed gene expression of curli fimbriae genes (*csgA* and *csgB*) (J. H. Lee et al., 2019). CsgD (master biofilm regulator) was significantly down-regulated by *B. monnieri*, *G. glabra* and *A. marmelos* (Fig. 19). Strikingly, pEtN-cellulose production has not been affected by a couple of plant extracts such as *B. monnieri*, *F. arabica*, *A. calamus* on macrocolonies, but they remarkably suppressed the gene expression of *dgcC*. It could be possible that even less concentration of DgcC is sufficient for normal activation of BcsA.

None of the plant extracts has any effect at the expression level of RpoS (master stress response regulator) (Fig 18) which means that downregulation of CsgD is not via RpoS stress response regulator. As per our previous data of growth curves and cfu/mL, it seems that in the presence of plant extracts, cells are either delaying or not entering to stationary phase of growth. So, it

could be highly possible that RpoS is being expressed not because the cells are entering the stationary phase, it could be because of the stress of nutrient deficiency around them, acidity, etc. It has already been documented that RpoS can be expressed even in the growing cells under series of different acute stress signals such as lack of nutrients, high osmolarity, acidity, radiation etc. (D. O. Serra & Hengge, 2014).

To further investigate the target for the downregulation of CsgD by *B. monnieri*, *G. glabra* and *A. marmelos* number of transcriptional factors required for regulation of CsgD were tested. None of the plant extracts have any effect on envelope stress response (via activation of RpoE and/or Cpx and /or Rcs) and heat shock response (Figure 20 and 21) which means, none of the above-said response regulators are involved in the suppression of CsgD by *B. monnieri*, *G. glabra* and *A. marmelos.* It is bit surprising to see that none of the plant extracts affected cell envelope stress responses as it is already investigated in previous studies that flavonoids and EGCG tend to trigger the effects from outside – both CsgA subunit assembly into amyloid fibers occurs at the bacterial cell surface (Evans & Chapman, 2014) and the signaling for activation of RpoE response is also initiated with some defects in protein folding in the cell envelope of Gram-negative bacteria (R. Hengge, 2019; Pruteanu et al., 2020; D. O. Serra et al., 2016).

So, it could be concluded that targeting at the genetic level is not as prominent as the extracytoplasmic anti-biofilm strategy is by plant extracts used in this project.

5.1.7 Effect on c-di-GMP, a key switch between growth and survival strategy

As the envelop stress responses are not involved in the downregulation of *csgD*, extracts of *B. monnieri*, *G. glabra,* and *A. marmelos* was further analyzed to see if c-di-GMP (major regulatory switch between motility and biofilm phase) are being targeted by these three selected plant extracts by using macrocolony with W3110 *pdeR dgcM* as a reference strain. Strikingly, data in this study suggest that none of the plant extracts are targeting c-di-GMP molecules (Figure 22). So, it has been found that downregulation of CsgD with *B. monnieri*, *G. glabra* and *A. marmelos* still needs to be uncovered as none of the tested targets such as c-di-GMP and stress responses (which are involved in regulating the expression level of CsgD) got affected by any plant extracts.

5.1.8 Promoting motility as an anti-biofilm strategy by *Bacopa monnieri*

Surprisingly, the flagellar gene cascade (*fliA* gene and *pdeH* gene) is being upregulated, nearly 2 times with *B. monnieri*. This is further supporting that cells are not entering to stationary

phase. The two lifestyles in bacteria are generally described; motile and sessile/biofilm, and during the transition from motile to biofilm, flagella undergo two types of variations; one is associated with short term, in which, flagella are functionally regulated to inhibit rotation or modulate the basal flagellar reversal frequency and in long term, flagella are likely diluted to extinction through growth (Guttenplan & Kearns, 2013). So, the upregulation of flagellar genes by *B. monnieri* is revealing that the extract of *B. monnieri* tends the *E. coli* to stay in the motile phase and thus, not allowing it to enter into the biofilm phase. This is an interesting anti-biofilm strategy that is not being extensively considered as a strategy to cope up with biofilm formation to date.

The target compound that would have involved in upregulating the flagellar gene expression could be affecting (i) the transcription of the *flhDC* operon itself or (ii) translation of *flhDC* mRNA, or (iii) proteolysis of FlhDC proteins. Factors involved in this regulation are known: e.g. the Csr system (in translation) or RflP/ClpXP protease (in proteolysis) which can be potential targets in promoting the flagellar cascade. Csr is a global regulatory system that utilizes CsrA (RNA binding protein), that can bind to mRNA of various genes, thus, affecting their structure, stability, translation, and/or transcription elongation (Potts, Leng, Babitzke, & Romeo, 2018). This system represses biofilm formation and activates cell motility and is mainly known for its post-transcriptional role in mRNA stability; can destabilize (*glgCAP*, *pgaABCD*, etc.) or stabilize (*flhDC*) the transcripts (Timmermans & Van Melderen, 2010).

So, motility promoting compounds could be interesting targets to inhibit biofilm formation and can be explored further.

5.1.9 Effects of plant extracts on pathogenic *E. coli* macrocolonies

Apart from testing the ten plant extracts on commensal *E. coli* K-12, these were further evaluated on pathogenic *E. coli* for their anti-biofilm activity on macrocolonies at two different temperatures, 28 deg. C and 37 deg. C (Fig. 11). In general, results are not as pronounced as in the case of UPEC and EAEC as in *E. coli* K-12. One possible reason could be the presence and absence of O-antigen that assists *E. coli* adhesion through hydrogen binding (Strauss, Burnham, & Camesano, 2009). Both EAEC and UPEC have O-antigen and tends to bind with each other and form aggregates (Strauss et al., 2009) and thus less surface area is available for the active compounds of accessing the cells to act on them OR only outer cells of the aggregates are exposed to the active compounds and inner cells are shielded from any such effect from active compounds. *E. coli* K-12 lacks o-antigen (Strauss et al., 2009) so its outer surface is more vulnerable for the active compounds to act on it. On the other hand, *B. monnieri*, *G. glabra* and

A. marmelos, colony morphologies were different in contrast to the untreated control which gives a clue that these plant extracts might have some active compound(s) that still have potential to involve at their molecular level and leading to some changes in their phenotypic characteristics.

So, pathogenic strains of *E. coli* may have evolved further to protect themselves from harmful compounds or environments.

5.2 Effects of plant extracts on growth and biofilm formation of *P. aeruginosa*

None of the plant extracts seems to inhibit the growth of *P. aeruginosa* at both 28 deg. C and 37 deg. C with 5 days of incubation on agar plates. *B. monnieri, G. glabra and F. arabica* promoted the growth, and colonies spread outward and covered nearly the whole 35 mm plate (Fig 12). Certain plant extracts such as *G. mangostana* and *T. chebula* seemed to promote biofilm formation as they stimulated the wrinkling of macrocolony biofilms, which is indicative of increased biofilm matrix production. This hyper-wrinkled phenotype was previously observed for mutants lacking phenazines, which are small diffusible redox-active molecules that balance the intracellular redox state and modulate colony biofilm morphology by inhibiting matrix production via the stimulation of c-di-GMP degradation under oxidizing conditions (Okegbe et al., 2017; Okegbe, Price-Whelan, & Dietrich, 2014) and with some purified flavonoids such as luteolin, quercetin, to a lesser extent myricetin which also promotes the wrinkling (Pruteanu et al., 2020). Because of the known anti-oxidative properties of plant compounds such as flavonoids, catechins, tannins etc. (Brunetti, Di Ferdinando, Fini, Pollastri, & Tattini, 2013; Grzesik, Naparło, Bartosz, & Sadowska-Bartosz, 2018; Maisetta et al., 2019), can interfere with the electron-shuttling activity of phenazines or can downregulate the production of phenazines (Pruteanu et al., 2020).

Interestingly, *B. monnieri*, *F. arabica*, and *A. marmelos* increasing the level of phenazines which can be easily concluded by the greenish colonies which may be an attempt to compensate for compromised phenazine activity. These plant extracts may have some anti-oxidative properties which may be facilitating the blockage of redox shuttling by the phenazines within the macrocolony biofilms towards which the cells are responding with higher production of phenazines (Pruteanu et al., 2020). Many of the plant extracts are not associated with a visually increase in green color production or in general, pyocyanin synthesis. *P. aeruginosa* cells can bind to eDNA in biofilms through the intercalation of pyocyanin and thus are involved in aggregation and biofilm formation (T. Das, Kutty, Kumar, & Manefield, 2013; Theerthankar Das & Manefield, 2012). Therefore, it could be possible that in other plant extracts the biofilms

are impaired via reduced production of the phenazine pyocyanin. For submerged biofilms, *B. monnieri, G. glabra*, and *A. marmelos* proved to be the best in completely eliminating the biofilm formation without affecting growth (Fig. 15). On the other hand, there are some plant extracts such as *C. zeylanicum* and *A. calamus* needs further study to understand the reasons for promoting biofilm formation of *P. aeruginosa*. Flavonoid, myricetin is already known for its biofilm stimulating effect of submerged biofilm formation (Pruteanu et al., 2020). So, it could be possible that these two plants are rich in myricetin.

Few plants extract such as *C. zeylanicum, G. mangostana,* and *T. chebula* produced brown pigment. It has already been documented long back ago that brown variants are spontaneous mutants occurring in the normal populations and they showed increased resistance to virulent bacteriophage E79 (Howarth & Dedman, 1964). Later it has been found that melanogenic strains are produced in clinical isolates of *P. aeruginosa* with the deletion of certain genes in the chromosomes in contrast to their parent strains (Hocquet et al., 2016). They also further showed that these brown pigment mutants are produced only in the biofilm communities *in vitro* as no brown strains were produced in planktonic in culture. They can outcompete their parent in a co-culture despite having impaired fitness. Their study also supported that these pyomelanin-producing mutants narrow down their substrate utilization profile in contrast to their counterparts i.e. free-living bacteria and produce fewer virulence factors *in vitro* (Hocquet et al., 2016).

In conclusion, *P. aeruginosa* biofilms are greatly affected by the plant extracts, some promoted biofilm production by producing wrinkled colonies and others produced higher phenazines and others did not show any visual green color phenazine. *B. monnieri* consistently completely eliminating the submerged biofilms of *P. aeruginosa* in addition to commensal *E. coli* K-12 and pathogenic *E. coli*. So, it could have some interesting active compound(s) which can interfere with biofilm formation on solid surfaces and submerged biofilm formation in Gram-negative bacteria irrespective of their differences in matrix composition and architecture.

5.3 Effects of plant extracts on growth, biofilm formation and viability of Gram-positive bacteria

Using two Gram-positive strains, one soil bacteria, *B. subtilis,* and another pathogenic strain, *Staphylococcus aureus,* plant extracts were tested for their broad range anti-biofilm activity. Surprisingly, *G. glabra* extract was completely toxic for both the tested Gram-positive strains on macrocolonies and submerged biofilms so it might have some antimicrobial compounds (Fig. 12 and 16). Isoflavonoids of *G. glabra* such as hispaglabridin and B, 4'-O-

methylglabrridin, glabridin, glabriol and 3-hydroxyglabrol are already known for their antibacterial effects (Irani, Sarmadi, Bernard, Ebrahimi Pour, & Shaker Bazarnov, 2010). *G. mangostana* also inhibited the growth of *B. subtilis* but only at 37 deg. C without showing any effect on growth at 28 deg. C on agar plates (Fig. 12). This shows that antimicrobial activity is temperature-sensitive in the case of *G. mangostana*. Antimicrobial activity of metabolite produced by *Weissella confusa* is also temperature-dependent, which is higher at low temperature (27 deg. C) and is reducing with increasing temperature (SERNA-COCK, VALENCIA H, & RUALES S, 2014). Mangostin, a xanthone from *G. mangostana* is already known to be highly active as an antimicrobial agent against *B. subtilis* and other pathogenic microorganisms such as *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Salmonella typhimurium* (Sundaram, Gopalakrishnan, Subramanian, Shankaranarayanan, & Kameswaran, 1983). Crude chloroform extract of mangosteen pericarp (fruit of *G. mangostana*) is also known for its antibacterial activity against various dental pathogens such as *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus salvarius*, *Streptococcus oralis,* and *Lactobacillus acidophilus* (Janardhanan, Mahendra, Girija, Mahendra, & Priyadharsini, 2017).

In this project, also, most of the plant extracts are not supporting the growth of *B. subtilis* in liquid culture except *C. zeylanicum*, *B. monnieri* and *A. marmelos* which were surprisingly promoting the growth on 96 well plates (Fig. 16). Not much is known if plants are producing any such active compounds that are supporting and promoting bacterial growth. Most interestingly, plant extracts of *B. monnieri* and *A. marmelos* which are promoting growth of *B. subtilis* were also significantly inhibiting biofilm formation (Fig 16). This could mean that do not only inhibit but rather let the cells stay in the liquid phase instead of adhering to the plastic plate as their OD is higher. A novel quinone, phenanthrenequinone, derived from ethyl acetate extract of *A. marmelos* has a concentration dependent anti-biofilm activity against *E. coli*, *Salmonella typhii* and *P. aeruginosa* respectively (Rejiniemon et al., 2014) so may be the same compound is active against Gram-positive species too. In case of *S. aureus*, nearly all the plant extracts tested in this study inhibited the growth whereas *G. glabra* and *T. chebula* are completely toxic for growth in liquid culture (Fig. 16).

Data in this study is supporting that Gram-positive bacteria are more sensitive towards the effect of active compounds in comparison to Gram-negative, it is because of the difference of their cell wall composition and structure. Cell wall of Gram-negative bacteria is a two-layered having one additional outer membrane which makes it resistant to many antibiotics including beta-lactams, quinilons, and other antibiotics so it is difficult for hydrophobic active compounds such as flavonoids to get access the targets inside the cell in contrast to Gram-positive which is

thick peptidoglycan single layer (Breijyeh, Jubeh, & Karaman, 2020). It has already been shown by various studies that Gram-positive bacteria are sensitive towards active compounds derived from plants in comparison to Gram-negative (Irani et al., 2010). Extract of *C. zeylanicum* is more effective against *Staphylococcus aureus* than *E. coli* (Salma et al., 2019). The degree of effectiveness of biologically active compounds also depends upon the type of solvent used for the extraction procedure, for instance, diethyl ether extracts of *B. monnieri* is potent antibacterial against Gram-positive *Staphylococcus aureus* ATCC 25923, ethyl acetate against Gram-negative *Escherichia coli* ATCC 25922 at higher concentrations and ethanoic extracts has antifungal activity against strains of *Aspergillus flavus*, and *Candida albicans* (Mkk, Sp, & Irfan, 2019). Antimicrobial effectiveness also depends on the part of plants used in extraction of biological activity as extract of roots and leaves from *G. glabra* showed antimicrobial activity against *Candida albicans* whereas ethanoic extracts of leaves are the most active against Gram positive bacteria (Irani et al., 2010).

Another interesting result is the biofilm promotion of *S. aureus* with *B. monnieri* which was eliminating the biofilm formation of both Gram-positive *B. subtilis* and Gram-negative *E. coli* (both commensal and pathogenic strains, EAEC and UPEC) and *P.aeruginosa* on 96 well plate. The most probable reason for this could be the interference of host factors (as the plates in this particular case were coated with fibronectin) involved and the reaction of compounds of extracts with fibronectin. This needs further study to understand the interactions of compounds with bacteria in the presence of host factors.

Most of the plant extracts used in this study are already known to have active compounds against various Gram-positive pathogenic strains, for instance, diethyl ether extracts of *B. monnieri* has potent antibacterial effect against Gram-positive *Staphylococcus aureus* ATCC 25923 and many other Gram positive bacterial species (A. Khan, Uddin, Shukla, Athar, & Khan, 2010; Mkk et al., 2019) and a compound extracted from *B. monnieri,* Bacoside A is also significantly removing the biofilm of *Staphylococcus aureus* (Parai et al., 2017), ethanolic extract of *G. glabra* and methanol extract of leaves of *A. marmelos* are active against *Staphylococcus aureus* (ATEŞ & TURGAY, 2003) (Poonkothai & Saravanan, 2008), *T. cordifolia* exhibited antimicrobial properties against *Streptococcus mutans* (Agarwal, Ramamurthy, Fernandes, Rath, & Sidhu, 2019), *C. zeylanicum* has antimicrobial activity against *Staphylococcus aureus* (Salma et al., 2019) and cinnamaldehyde extracted from *C. zeylanicum* also showed antibiofilm activity against *Streptococcus pyogenes* (Firmino et al., 2018), chloroform extract of mangosteen pericarp (fruit of *G. mangostana*) showed an effective zone of inhibition against *Streptococcus mutans, Streptococcus sanguis, Streptococcus*

salivarius, Streptococcus oralis and Lactobacillus acidophilus (Janardhanan et al., 2017) and an active compound from peels of fruit of *G. mangostana*, alpha-mangostin is significantly reducing biofilm (by killing the bacteria in biofilm) of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (Phuong et al., 2017), extracts of *F. arabica* and *A. calamus* are also potent antibacterial against *Staphylococcus aureus* (Abobaker, 2017; Rita, Swantara, & Utami, 2019). All this literature is further supporting that Gram-positive are more sensitive in comparison to Gram-negative bacteria.

Many plant extracts are also known to have antibacterial effects against bacteria causing oral infections such as caries and periodontitis and involving in oral biofilms (Agarwal et al., 2019; Janardhanan et al., 2017; Saliem & Abedsalih, 2018; Saxena, Lakshminarayan, Gudli, & Kumar, 2017). So, it could be concluded that a combination of such active compounds from such plant extracts can be used in combination or ratios to develop a mouth rise/mouth wash in the prevention of dental caries or treating some oral infections.

To summarize this, Gram-positive bacteria are more sensitive to active compounds in plant extracts than Gram-negative. Few plant extracts such as *B. monnieri, F. arabica* and *A. marmelos* are promoting the growth of *B. subtilis* (Gram-positive) on agar plates and significantly inhibiting the biofilm formation. These results are clearly showing that plant extracts have both antibacterial and anti-biofilm compounds that could be explored for future studies and treatment techniques.

5.4 What could be the active compounds in the plant extracts that interfere with bacterial biofilm formation?

Hundreds of compounds from plant origin are already known to have various biological activities (Silva et al., 2016). Plants used in this study have many known compounds for their various biological activities such as antimicrobial, anti-biofilm, neuroprotective, etc. The focus of this project is to explore biofilm inhibiting plants/compounds having some neuroprotective activities (as the selection of plants used in this study was based on the rationale that plants used in traditional medicine against neurodegenerative diseases involving amyloid fiber/plaque formation may contain anti-amyloidogenic compounds). Table 7 listed some of the already known compounds from previous studies of the plant origin used in this study having both anti-biofilm and neuroprotective activities.

In *B. monnieri*, already seven active anti-biofilm compounds are known to have anti-biofilm activity (listed in Table 7) such as bacoside A, ursolic acid, luteolin, quercetin, umbelliferone,

catechin, and apigenin against various Gram-positive and Gram-negative bacteria. So, it could be concluded that plant extracts are not containing cocktails of active compound that show anti-biofilm activity against multiple bacterial species. In this project, however, two purified compounds, Bacoside A from *B. monnieri* and Glabridin from *G. glabra* did not completely show as strong effects as we observed with a whole plant extract (Fig. 28 and 29). Among the known compounds in B. *monnieri*, ursolic acid is interesting as it is known to induce motility in *E. coli* K-12 (Dacheng Ren et al., 2005) and something similar was also observed in this project also Fig. 23 (upregulation of FliA and PdeH). Dacheng Ren and his co-workers analyzed DNA microarrays to study the gene expression profile of *E. coli* K-12 with and without ursolic acid and found that 19 genes were consistently induced both at low and high concentrations of ursolic acid. Gene responsible for the motility, *motAB* is one among them (Dacheng Ren et al., 2005). They further proved that low mobility is favorable for conjugation which further promotes biofilm development. Ursolic acid decreases biofilm formation by inducing expression of *motAB*, making cells too motile to stay in the biofilm community stably, whereas deleting *motAB* gene simply neutralizes the ability of ursolic acid to inhibit biofilms since the cells are paralyzed (Dacheng Ren et al., 2005).

Bacoside A has also been tested in this study against *E. coli* K-12 Fig. 29 but nothing much interesting has been observed against *E. coli* K-12. But it has already proven to be actively inhibiting the biofilm formation of *S. aureus* and *P. aeruginosa* (Parai et al., 2017). *B. monnieri* seems to be inhibiting the biofilm formation of both *S. aureus* and *P. aeruginosa* on macrocolonies but on microtiter plate, it is promoting biofilm formation of *S. aureus*. It could be because of the complex interactions of bacteria and *B. monnieri* extracts with fibronectin-coated 96 well plate. Similarly, phenanthrenequinone (novel quinone) derived from *Aegel marmelos* is known to be actively inhibiting the biofilm formation of *E. coli* and *P. aeruginosa* (Rejiniemon et al., 2014) so it could be one such active compound for seeing inhibition of biofilm formation with *A. marmelos*.

It could be summarized that plants have multiple active biofilms inhibiting compounds that could target through different molecular pathways and/or active against different microbial species. The plants may evolve to target multiple microbial species, trying to colonize their surfaces under different physiological environmental conditions throughout the year.

5.5 Why and how can plant extracts with alleged memory-promoting or anti-neurodegenerative effects also inhibit biofilm formation of enteric bacteria?

The logical thought behind this project were the following ecophysiological considerations:

- Plants have to defend themselves against bacteria colonizing their surfaces-should have efficient anti-biofilm compounds
- Plants used in traditional medicine against chronic infections should have potent anti-biofilm compounds
- Plants used in traditional medicine against neurodegenerative diseases involving toxic amyloid fiber/plaque formation may contain anti-amyloidogenic compounds that could also interfere with the productions of biofilm matrix which for the most bacteria contain functional amyloid fibers (such as curli fibers in *E. coli*)

In addition to this, enteric bacteria spend part of their life cycle in the environment including on plant surfaces. For instance, *E. coli* O157:H7 is associated with green leafy vegetables and causes gastroenteritis with the intake of contaminated leafy vegetables (Fink et al., 2012). Plant associated microorganisms or insects facilitate the survival and transmission of enteric pathogens in plants where they successfully adhere and invade plants under harsh environmental conditions (Lim, Lee, & Heu, 2014). Based on microarray performed on *E. coli* K-12 and O157: H7, it has been shown that bacteria tend to attach the plant surfaces by curli fibers and pEtN-cellulose (Fink et al., 2012; Lim et al., 2014). Plants can perceive the human pathogens and trigger the plant innate immunity to ward off microbial invaders (Melotto, Panchal, & Roy, 2014). Targeting matrix components is easy for plants to access as they do not have to cross the barrier of the cell wall and cell membrane (Pruteanu et al., 2020; D. O. Serra et al., 2016). Amyloid fibers are abundant and highly diverse in many phyla including enteric and oral bacteria (Larsen et al., 2007; Oli et al., 2012). Although there are differences at the level of sequences of their protein subunits yet the supermolecular structure is quite similar (R. Hengge, 2019). Such classical supermolecules of amyloid fibers and plaques are also found in patients suffering from neurodegenerative disorders such as Alzheimer's disease where they are toxic (R. Hengge, 2019). So, such plants that can disrupt beta-amyloid fibers in bacterial species can also be beneficial for the patients suffering from neurodegenerative disorders such as Alzheimer's and Parkinson disease or vice versa (Table 7). This rational was the base for the selection of plants in this project. And results of this study are fully supporting and shows the success of this strategy of selection of plants. In one previous study, it has been highlighted that most of the plant flavonoids are glycosylated and enteric bacterial attempt to use sugar moieties as nutrients may actually activate flavonoids to reduce the curli fibers and inhibit the bacterial adherence and colonization (Braune & Blaut, 2016; Pruteanu et al., 2020)). Such activated flavonoids can potentially interfere with the production of curli by the gut microbiome. Initially, curli were thought to be expressed at temperature less than 30 deg. C (ambient temperature),

now it has been seen that it is the growth medium that actually supporting the biofilm formation at 37 deg. C in commensal *E. coli*, EAEC, UPEC, and other avian pathogenic *E. coli* (Van Gerven, Van der Verren, Reiter, & Remaut, 2018). In 2015, it has already been shown that biofilm-producing curli fibrils by enteric bacterial infections are contributing to the progression of autoimmunity disease such as systemic lupus erythematosus (SLE) (Gallo et al., 2015) and also responsible for inflammation (Tükel et al., 2009). So, consumption of plants containing amyloid targeting compounds such as flavonoids, EGCG was already known in previous studies (Pruteanu et al., 2020; D. O. Serra et al., 2016) could be beneficial as an anti-inflammatory and could also be a target for the treatment of autoimmunity diseases such as systemic lupus erythematosus. As mentioned earlier, oral biofilms contain bacterial amyloids e.g. produced *Streptococcus mutans* which is kind of the ‚lead bacterium' in the complex oral biofilms (Oli et al., 2012). EGCG alone or along with materials used in the treatment of tooth infections has produced promising results by impairing the adherence and colonization of *S. mutans* and caries formation (R. Hengge, 2019). Consumption of plants rich in amyloid targeting compounds are in general healthy for our overall, intestinal, milieu. As per the results obtained in this study, some of the tested plants extracts are inhibiting the biofilm formation of tested enteric and other pathogenic bacterial strains such as EAEC, UPEC, *P. aeruginosa,* and *S. aureus.*

So, it could be concluded that consumption of plants having high content of anti-biofilm compounds most specifically targeting amyloid fibrils in general is beneficial for overall human health as these have an impact on the pathophysiology of neurodegenerative diseases and on memory and cognitive performance, can improve gut and oral health by reducing the inflammation caused by amyloid produced by enteric and oral bacteria and lastly, such compounds are also affecting the biofilm formation of bacteria involving in chronic infections.

5.6 Conclusions and Perspective

All the plant extracts affected the biofilm formation of commensal *E. coli* K-12 by interfering in the assembly of amyloid subunits directly. Few also affected the biofilm-related genes to control biofilm formation. There is not a single plant extract that is showing broad-spectrum effect i.e. on different biofilms produced by different bacterial species or different strains of same species except *B. monnieri*, which showed promising results against all biofilms except with *Staphylococcus aureus,* where it seems to promote submerged biofilm which could be a technical defect because of the interaction of complex mixture of extract with human fibronectin that coated the plates. This is further suggesting that though these extracts seem to

be promising at on plastic plates, they might not be effective *in vivo* where host components are also involved to produce biofilms so further study considering human factors needs to be investigated.

Moreover, Table 7 shows that there is not one single 'magic compound' that can effectively work against diversified biofilm compositions and structures produced under different physiological conditions (for instance at different temperatures, growth media, etc.) by same strain or different bacterial species under same conditions; for instance, *B. monnieri* (Table 7) itself has many known compounds which individually can be effective anti-biofilm agents against different bacterial species, which is further supporting that plants use cocktails of compounds rather than single compounds to combat different bacterial biofilms produced by different species. A combination of plant extracts could be used for the treatment of infections where more than one species is associated with one single infection e.g. chronic wounds, in which *P. aeruginosa* and *S. aureus* make producing mixed biofilms, for instance, extract of *B. monnieri* (strong anti-biofilm effect against *P. aeruginosa*) in combination with *G. glabra* (strong antibacterial against *Staphylococcus aureus*) could be good candidates to deal with it. Though *B. monnieri* is promoting the submerged biofilm of *Staphylococcus aureus* but in the presence, of *G. glabra* it would not be able to grow and produce any biofilm. So, a strategy of combining anti-biofilm extract along with antibacterial may actually work. There are still lots of gaps such as considering the role and interactions of the host factors in the biofilms in the presence of anti-biofilm agents, interactions of bacteria in a co-biofilms, and their overall resistance/tolerance towards anti-biofilm compounds in mixed culture and responses towards a combination of plant extracts that needs to be addressed.

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DECLARATION

I hereby declare that this Ph.D. project on title "**Anti-biofilm activity of plants used in Ayurvedic medicine and their molecular mechanisms of actions on *E. coli* biofilms**" has been done by me independently under the supervision of Prof. Dr. Regine Hengge, at the Institute of Microbiology/Biology, Humboldt-Universität zu Berlin, Germany. The information derived from literature has been acknowledged in the text and the list of references provided. No part of this work was previously presented for another degree or diploma at this or any other institution.

Amita Bhatti (Signature) DATE: ----------